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*Bacillus stearothermophilus*(  
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**STUDIES ON  
STRUCTURE AND FUNCTION  
OF  
THERMOSTABLE ALANINE RACEMASE  
FROM  
*Bacillus stearothermophilus***

**HIROHIDE TOYAMA  
1992**

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## INTRODUCTION

Alanine racemase (EC 5.1.1.1) occurs widely in prokaryotes. The enzyme requires pyridoxal 5'-phosphate (PLP) as a coenzyme, and catalyzes racemization of D- and L-alanine (1). The D-enantiomer, as well as L-enantiomer, is used for the synthesis of the peptidoglycan layer of bacterial cell walls as an essential component (1). Alanine racemase is also a key enzyme for D-amino acid metabolism. Therefore, the enzyme has been studied as a target of various natural and synthetic antibiotics such as D-cycloserine (2),  $\beta$ -chloro-D-alanine (3, 4), and (1-aminoethyl)phosphonate (5, 6). The reaction mechanism and the enzyme structure also have been investigated in some detail (7). The amino acid sequences of alanine racemases from *Salmonella typhimurium* ((8) for the DadB enzyme; (9) for the Alr enzyme) and *Bacillus subtilis* (10) were determined from the DNA sequence of their genes. Recently, Inagaki *et al.* have cloned the gene for thermostable alanine racemase from *B. stearothermophilus* and purified the enzyme (11), and Tanizawa *et al.* determined its primary structure (12). The enzyme is currently under crystallographic investigation to reveal the three-dimensional structure (13).

The sequences of four alanine racemases display considerable homology (about 33% homology on average for their sequences), and the *B. stearothermophilus* enzyme shows 35 and 31% sequence homology with DadB and Alr enzymes, respectively. Furthermore, local sequences including the active-site and hinge regions are well conserved. An octapeptide containing the lysyl residue that binds PLP is conserved in all the four enzymes (12). Amino acid racemase with broad substrate specificity of *Pseudomonas putida* (EC 5.1.1.10) contains the same sequence (14). Both the *B. stearothermophilus*



enzyme and the broad-specificity amino acid racemase (15) consist of two identical subunits. In contrast, two alanine racemases, DadB (8) and Alr (9) of *S. typhimurium* and the *Streptococcus faecalis* enzyme (6) occur as a monomer. Therefore, in Chapter 1, the author studied dissociation and unfolding of the *B. stearothermophilus* enzyme, and describe that the enzyme dissociates into monomers in the presence of about 1.5 M guanidine hydrochloride (GdnHCl), and that the monomeric enzyme is inactive.

Galakatos and Walsh (16) reported that both DadB and Alr isozymes of alanine racemase of *S. typhimurium* suffered from limited proteolysis at two homologous positions by trypsin, chymotrypsin, or subtilisin, generating two non-overlapping polypeptides with  $M_r$  28,000 and 11,000, and a tetrapeptide, which was regarded as a hinge region. The DadB enzyme proteolyzed with chymotrypsin behaved as an associated form of two fragments, exhibited a far-UV CD profile identical with that of the native enzyme, and possessed 3% of the original catalytic activity. They also showed that the controlled subtilisin digestion of the thermostable enzyme of *B. stearothermophilus* gave a fragmentation pattern similar to that derived from Alr and DadB enzymes on SDS-PAGE. However, they had no comments about the activity of the thermostable alanine racemase after it was nicked. The author found that the nicked form of thermostable alanine racemase is still active, as described in Chapter 2.

A larger protein usually has structural domains which correspond to folding units in the parental polypeptide chain (17). To examine whether the two polypeptide fragments generated by limited proteolysis correspond to structural domains, the author attempted to prepare the two polypeptides by means of genetic engineering in Chapter 3. The author constructed a mutant

alanine racemase gene of *B. stearrowthermophilus*, which contains a new set of termination and initiation codons in the position of the gene corresponding to the putative hinge region of DadB and Alr racemases. The gene was expressed as an active alanine racemase composed of two dissimilar polypeptides. A single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli*, but no protein reactive with the antibody against the wild-type alanine racemase was produced. Therefore, it is suggested that the two polypeptide fragments can fold into an active structure only when they are co-translated and that they correspond to structural folding units in the parental polypeptide chain.

In Chapter 4, the author compared the denaturation process of the fragmentary enzyme to that of the wild-type enzyme. It is revealed that the fragmentary enzyme is more labile to the GdnHCl, especially at low concentration.

The last Chapter, the author isolated the each fragment from the fragmentary enzyme corresponding to the domains of the wild-type enzyme, in sufficient amounts to study their folding in vitro. The author described the reconstitution of the fragmentary alanine racemase by folding of the two fragments corresponding to the putative domains.

# CHAPTER 1

## Subunit Dissociation and Unfolding of Alanine Racemase of *Bacillus stearothermophilus*

Alanine racemase genes were cloned from *Salmonella typhimurium* (8, 9), *Bacillus stearothermophilus* (11) and *B. subtilis* (10), and sequenced (12). The sequences of four alanine racemases display considerable homology: about 33% homology on average for their sequences. An octapeptide containing the lysyl residue that binds PLP is conserved in all the four enzymes (8, 9, 10, 12). Amino acid racemase with broad substrate specificity of *Pseudomonas putida* (EC 5.1.1.10) contains the same sequence (14). Both the *B. stearothermophilus* enzyme and the broad-specificity amino acid racemase (15) consist of two identical subunits. In contrast, two alanine racemases, DadB (8) and Alr (9) of *S. typhimurium* and the *Streptococcus faecalis* enzyme (6) occur as a monomer.

The autor have studied dissociation and unfolding of the *B. stearothermophilus* enzyme to show the inactivity of its monomeric form. The autor characterized intermediates between the native and the denatured forms by circular dichroism (CD) and fluorescence spectroscopies, and by examination of the enzyme activity and hydrodynamic properties.

The autor here describe that the enzyme dissociates into monomers in the presence of about 1.5 M guanidine hydrochloride (GdnHCl), and that the monomeric enzyme is inactive.



# MATERIALS AND METHODS

## *Materials*

Superose 12 packed column ( $\phi 1 \times 30$  cm) was purchased from Pharmacia; MW-Marker (marker proteins for gel filtration) and cross-linked cytochrome *c* oligomers were from Oriental Yeast, Osaka, Japan; GdnHCl (specially prepared reagent grade) was from Nacalai Tesque, Kyoto, Japan. Alanine racemase was purified from recombinant *E. coli* cells carrying a plasmid coding for the enzyme gene of *B. stearothermophilus* as described previously (11, 13). Alanine dehydrogenase of *B. stearothermophilus* was also purified from recombinant *E. coli* cells as described previously (18). The other chemicals were of analytical grade.

## *Enzyme and Protein Assays*

The enzyme was routinely assayed in the D- to L-alanine direction by coupling with the L-alanine dehydrogenase reaction. The reaction mixture (1 ml) contained 100 mM glycine-KOH buffer (pH 9.0), 30 mM D-alanine, 2.5 mM NAD<sup>+</sup>, 10 units of L-alanine dehydrogenase, and an appropriate amount of alanine racemase, and the reaction was performed at 50 °C and followed spectrophotometrically with an increase in absorbance at 340 nm due to the NADH formation.

The enzyme activity in the presence of GdnHCl was measured by optical resolution of D-alanine produced from the L-isomer by high performance liquid chromatography followed by fluorometric determination of their amounts. The reaction mixture contained 100 mM L-alanine, 50 mM

potassium phosphate buffer (pH 7.5), 0.01% 2-mercaptoethanol, and 10  $\mu$ g of enzyme in a final volume of 1 ml. After incubation at 25 °C for 5 min, 10  $\mu$ l of the mixture was mixed with 90  $\mu$ l of 5% trichloroacetic acid, and the precipitate formed was removed by centrifugation. A 20- $\mu$ l aliquot of the supernatant solution was applied to a Cosmosil 5C18-AR column ( $\phi$ 4.6 x 150 mm, Nacalai Tesque) with an elution buffer (pH 5.5) containing 2 mM Cu<sup>++</sup>-complex of *N,N*-di-*n*-propyl-L-alanine (19) at a flow rate of 0.5 ml/min. D-Alanine eluted was analyzed with an on-line fluorophotometer with *o*-phthalaldehyde as a post-column reagent (20).

One unit of alanine racemase was defined as the amount of enzyme that catalyzed the formation of 1  $\mu$ mol of L-alanine per min. Protein concentration was measured by the method of Bradford (21).

### *Spectrophotometric Measurements*

CD measurements were carried out with a JASCO J-600 recording spectropolarimeter at 25 °C with a 1-cm light path length cell. The instrument was calibrated with (+)-10-camphorsulfonic acid,  $\epsilon = +2.37 \text{ M}^{-1} \text{ cm}^{-1}$  at 290.5 nm. In calculation of the mean residue ellipticity  $[\theta]$ , the mean residue weight was taken to be 112. for the enzyme protein. The CD spectra were obtained at protein concentrations of 0.02 to 0.1 mg/ml in the far UV region (215 to 240nm) under nitrogen atmosphere. Fluorometric measurements were carried out with a Hitachi MPF-4 spectrofluorophotometer.

All experiments for dissociation and unfolding of alanine racemase by GdnHCl were done in 1 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol. The ellipticity at 222 nm and fluorescence were

measured after they had reached a constant value on incubation at 20 °C for 10 min.

### ***Renaturation***

The enzyme was incubated with 6M GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol at 37 °C for 30 min. The GdnHCl concentration in the denatured enzyme solution was reduced to 60 mM by dilution with 10 mM of the buffers supplemented with 0.01% 2-mercaptoethanol and 10  $\mu$ M PLP: potassium phosphate, pH 7.0 and 7.9; Tricine-KOH, pH 8.3, 8.5 and 9.0; Glycine-KOH, pH 9.1, 9.7, 9.8 and 10.3; K<sub>2</sub>HPO<sub>4</sub>-KOH, pH 10.5 and 11.5. The enzyme activity was not affected by 60 mM GdnHCl.

### ***Gel Filtration***

Gel filtration chromatography was done with a Pharmacia Fast Protein Liquid Chromatography (FPLC) system equipped with a Superose 12 column ( $\phi$ 1 x 30 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol, 0.2 M KCl and various concentrations of GdnHCl. Protein elution was monitored by measurement of the absorbance at 280 nm. For estimation of molecular weight, a MW-Marker (Oriental Yeast) was used for gel filtration in the absence of GdnHCl; monomer, dimer, trimer, and hexamer of cross-linked cytochrome c were used in the absence or presence of GdnHCl.



## RESULTS

### *Reversible Denaturation of Alanine Racemase*

When the enzyme was incubated with L-alanine in 6 M GdnHCl at 37 °C for 30 min, D-alanine was not produced: the enzyme is inactive in 6 M GdnHCl. The inactivated enzyme was reactivated by dilution with 100 volumes of buffers (pH 9.0 - 10.5) to various extents according to pH of the buffers used (Fig. 1). The highest recovery was achieved around pH 10.0: about 75% of the original activity was recovered. However, no reactivation occurred below pH 8.5. When the denatured enzyme was dialyzed against 100 volumes of 50 mM Tris-HCl (pH 10.0) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol, about 76% of the original activity was recovered. Thus, the denatured enzyme is renatured either by dialysis or dilution to reduce the GdnHCl concentration.

### *Spectrophotometric Analysis of the Denaturation Process*

The absorption spectrum of the native alanine racemase exhibits an absorption maximum at 420 nm in the visible region due to the aldimine linkage formed between the  $\epsilon$ -amino group of Lys39 and PLP (12). The peak at 420 nm was shifted to 390 nm with an increase in GdnHCl concentrations (Fig. 2). This indicates that the aldimine linkage bond is hydrolyzed to produce the aldehyde form of PLP by the addition of GdnHCl. No further appreciable change in the spectrum was observed above 1.6 M GdnHCl: PLP occurs in an aldehyde form under the conditions (Fig. 2).



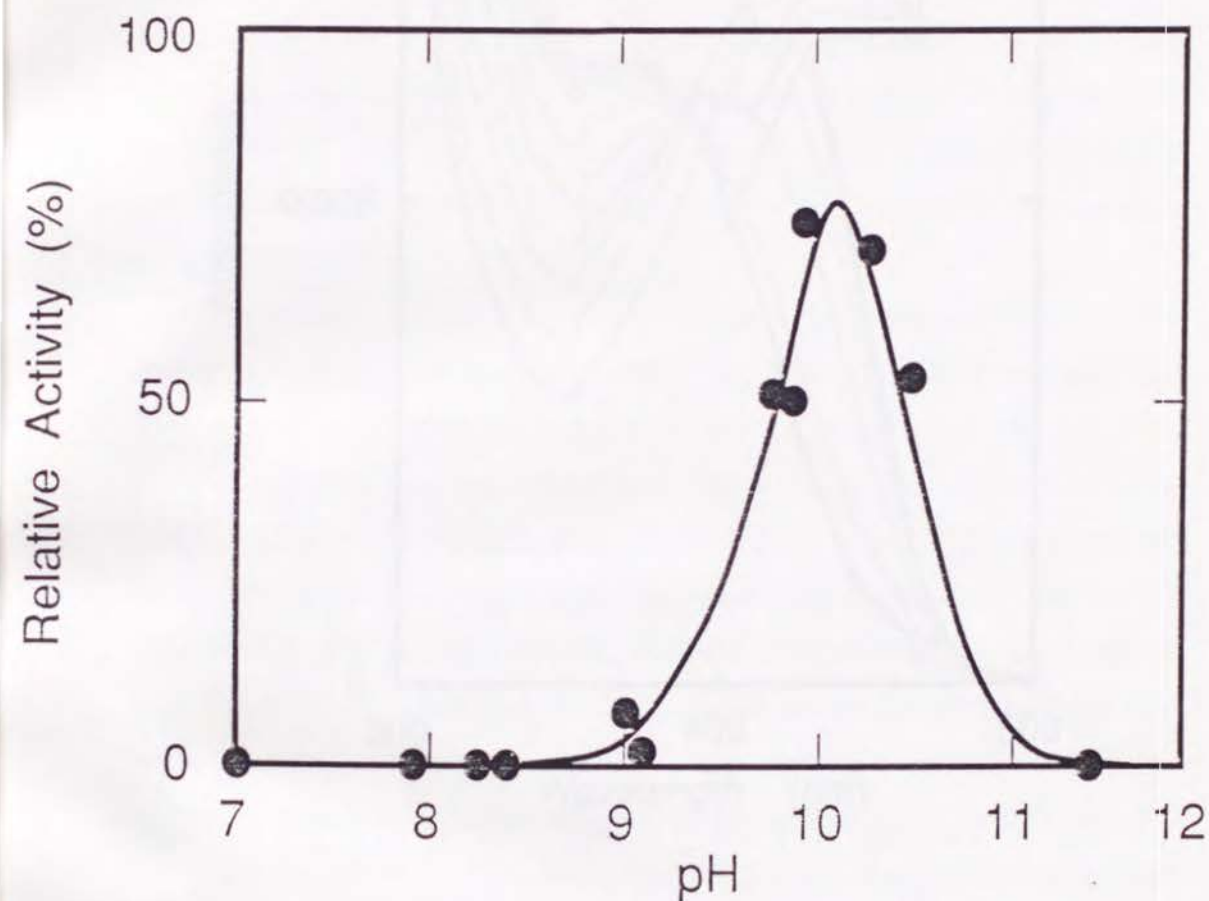


Fig. 1. Effect of pH on the renaturation of alanine racemase. The enzyme denatured with 6.0 M GdnHCl was renatured by 100-fold dilution with various kinds of buffers as described in Materials and Methods.

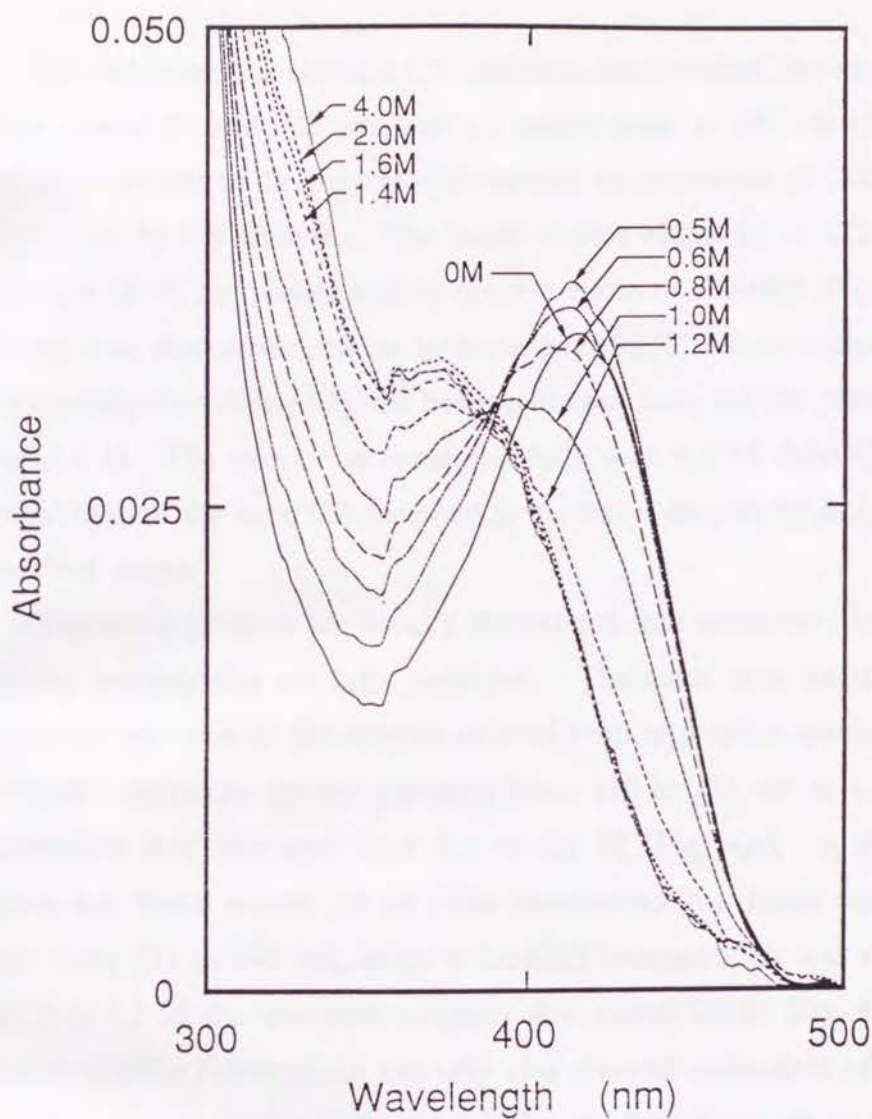


Fig. 2. Effect of GdnHCl concentrations on absorption spectra of the enzyme-bound PLP. The enzyme (final concentration, 0.18 mg/ml) was incubated with indicated concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then the absorption spectrum was measured.

The native enzyme shows a CD spectrum characterized by two negative minima around 21 and 222 nm, and a positive peak at 195 nm (12). The denaturation process of the enzyme with various concentrations of GdnHCl was followed by CD analyses. The mean residue ellipticity at 222 nm was measured at 20 °C and plotted against concentrations of GdnHCl (Fig. 3). The ellipticity was decreased with an increase in GdnHCl concentrations. The transition midpoints defined by the horizontal base lines and the plateau were around 2.0 M. The enzyme is denatured fully with 6.0 M GdnHCl, and is renatured to show the same CD spectrum as the native enzyme by dialysis done as described above.

Oligomeric proteins are usually dissociated into monomers before the consisting polypeptides are fully unfolded. The autor have examined the fluorescence spectrum of the enzyme derived from tryptophan residues. The fluorescence maximum moved gradually from 336 to 331 nm as a GdnHCl concentration was increased from 0.6 to 1.6 M (Fig. 4A). A transition midpoint was found around 1.0 M. The fluorescence maximum was shifted sharply from 331 to 348 nm, when a GdnHCl concentration was increased from 1.6 to 2.5 M: the transition midpoint was around 2.0 M (Fig. 4A). The change in relative fluorescence intensity also showed occurrence of the two transitions with a midpoint at about 1.0 M for the first phase and at about 2.0 M for the second phase (Fig. 4B). When a 3-fold lower concentration (0.03 mg/ml) of the enzyme was used in this fluorophotometric measurement, the transition midpoint of the first phase was observed at a lower concentration (about 0.8 M) of GdnHCl (data not shown), suggesting the presence of an enzyme concentration-dependent equilibrium of the subunit dissociation.

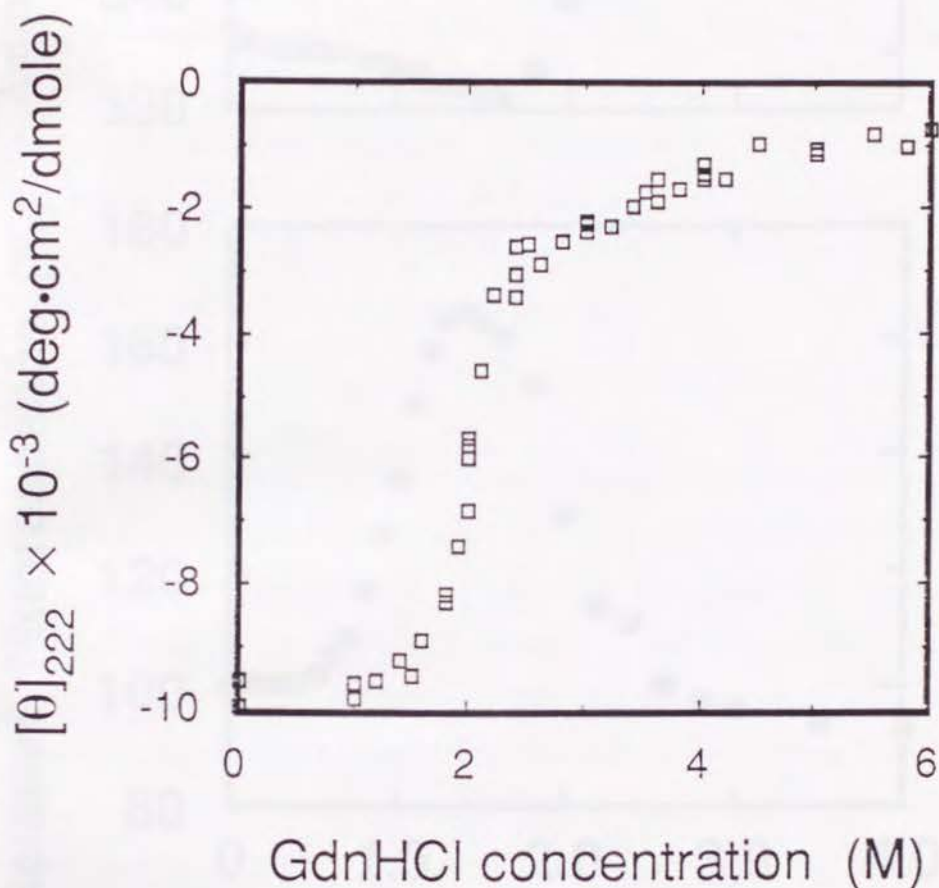


Fig. 3. Effect of GdnHCl concentrations on mean residue ellipticity at 222 nm of the enzyme. The enzyme (final concentration, 0.1 mg/ml) was incubated with various concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then CD was measured.



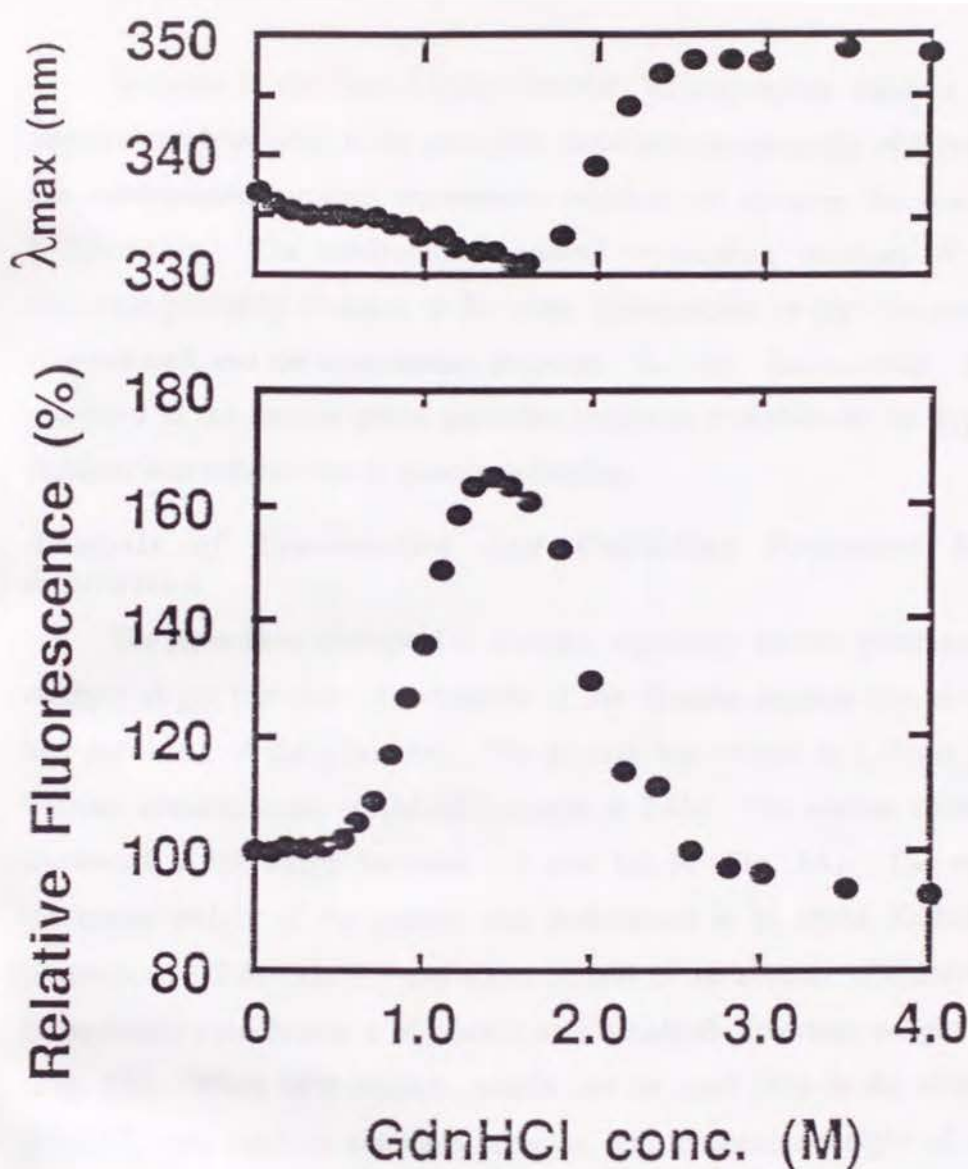


Fig. 4. Effect of GdnHCl concentrations on fluorescence spectra of the enzyme. The enzyme (final concentration, 0.1 mg/ml) was incubated with various concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then the fluorescence spectrum by excitation at 280 nm was measured. A, wavelength of the fluorescing maximum was plotted against GdnHCl concentrations. B, relative fluorescence intensity at 331 nm was plotted against GdnHCl concentrations.

Increase in the fluorescence intensity of tryptophan residues and the concomitant blue shift of the emission maximum are generally observed when the environment around tryptophan residues in proteins becomes more hydrophobic. The environment around tryptophan residues of alanine racemase probably changed to be more hydrophobic in the first transition. The red shift and the concomitant decrease in the fluorescence intensity observed in the second phase probably indicates exposure of the tryptophan residues into solvent due to protein unfolding.

### ***Analysis of Dissociation and Unfolding Processes by Gel Filtration***

The autor have attempted to examine separately the two processes by the method of gel filtration: dissociation of the dimeric enzyme into monomers and unfolding of the monomer. The protein was eluted as a single peak at various concentrations of GdnHCl except at 2.4M. The elution volume was increased in the range between 1.2 and 1.8 M (Fig. 5A). The apparent molecular weight of the protein was determined to be about 30,000 in the presence of 1.2 M GdnHCl and about 52,000 in the absence of GdnHCl with cross-linked cytochrome c oligomers as a standard molecular weight marker (Fig. 5B). When MW-Marker, which can be used only in the absence of GdnHCl, was used as standard proteins, the molecular weight of alanine racemase was shown to be about 80,000 (11). Therefore, the enzyme is dissociated into a monomeric form in the presence of 0.6-1.2 M GdnHCl. This range of GdnHCl concentration is very similar to that observed in the tryptophan fluorescence change (the first phase). Two peaks appeared in the presence of 2.4 M GdnHCl. The first peak probably shows an aggregated form of the enzyme, and the second represents a monomeric form of the

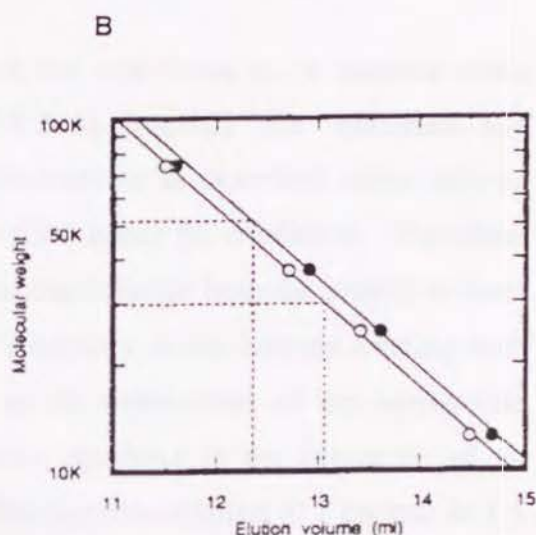
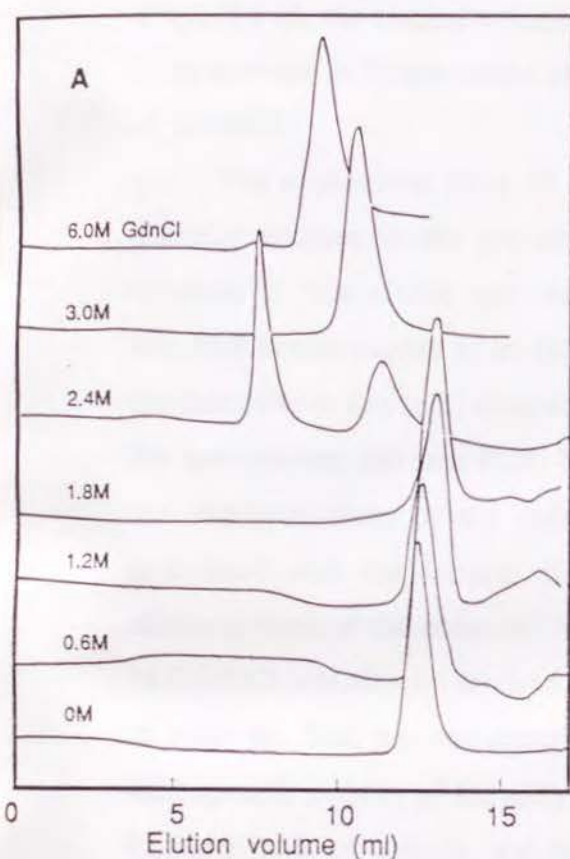


Fig. 5. Superose 12 gel filtration column chromatography of alanine racemase in the presence of various concentrations of GdnHCl. (A) Elution profiles. The enzyme (final concentration, 0.1 mg/ml) was incubated with indicated concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 30 min, then 50  $\mu$ l of the solution was applied to Superose 12 column as described in Materials and Methods. (B) Determination of the molecular weight of alanine racemase using cross-linked cytochrome c oligomers as standard proteins in the absence (●) or presence (1.2 M)(O) of GdnCl.



enzyme, although the elution volume was much decreased as compared with those at lower GdnHCl concentrations. As the concentration was increased above 3.0 M, the elution volume was decreased further. This is probably due to an increase in Stokes radius of the protein unfolded at higher concentrations of GdnHCl.

The monomeric form of the enzyme was found to be inactive when directly assayed in the presence of 1.5 M GdnHCl (see "Materials and Methods"). The visible spectrum of the enzyme as described above showed that PLP occurs mostly in an aldehyde form under the conditions. Therefore, the inactivity is due to 1) dissociation of the cofactor from the protein to form the apo enzyme and free PLP, 2) low reactivity of the enzyme binding with the aldehyde form of the cofactor, or 3) destruction of the active site associated with the subunit dissociation resulting in the formation of an aldehyde form of the cofactor. When the enzyme solution (0.1 mg/ml) in 1.5 M GdnHCl was directly taken to the assay mixture, in which PLP was omitted, to make the final enzyme concentration 0.12 nM, the enzyme showed about 40% specific activity of the native enzyme. The holo enzyme binds 1 mole of PLP per mole of subunit, and the apo enzyme shows an apparent Michaelis constant of 1.5-2.0  $\mu$ M for PLP (11). The final PLP concentration reached 0.23 nM by the dilution, and the enzyme should show little activity upon complete dissociation of PLP. However, this was not the case. The monomeric form of the enzyme still binds the cofactor through some other linkage than the aldimine bond in the presence of 1.5 M GdnHCl. Thus, the inactivity of the monomeric form of the enzyme is possibly due to the abnormal mode of binding of the cofactor. However, it is more conceivable



that the active site is destructed upon dissociation into monomeric forms to make the enzyme inactive as discussed below.

## DISCUSSION

Alanine racemase of *B. stearothermophilus* is unfolded by treatment with GdnHCl through two detectable phases: phases 1 and 2 were observed by fluorescence spectroscopy and gel filtration, and phase 2 by CD. The two phases probably are derived from the following transitions: Phase 1, dissociation of the dimer to monomers; Phase 2, unfolding of the monomer.

The monomeric form of the protein produced in Phase 1 is catalytically inactive. The absorption spectrum of the monomeric form showed that the carbonyl group of PLP is not in an internal Schiff base linkage. The reactivity of the carbonyl group of PLP is elevated by formation of a Schiff base with the  $\epsilon$ -amino group of a lysine residue at the active site of PLP-dependent enzymes (22). However, it seems unlikely that the inactivation is only due to the absence of the aldimine linkage. The mutant enzymes of aspartate aminotransferase (23) and D-amino acid aminotransferase (24) in which the PLP-binding lysine residue is replaced by arginine do not react with PLP to form a Schiff base, but they show a slight, but distinct activity. The inactivity of the monomeric form is mostly attributable to a local and minor structural change which occurs simultaneously with the subunit dissociation, but is not detectable by CD.

Thermostable alanine racemase undergoes a major structural change in Phase 2. The unfolded monomer non-specifically aggregates with each other

to form a species with a high molecular weight. Similar aggregation of isolated monomers have been reported for  $\beta_2$ -subunit of tryptophan synthase (25) and aspartate aminotransferase of *E. coli* (26).

The subunit dissociation is usually accompanied by destruction of the secondary structures when the autor use thermolabile proteins. The autor have succeeded to discriminately examine the subunit dissociation and the protein unfolding by means of a thermostable enzyme. Thermostable alanine racemase is probably a feasible material to study the mechanism of subunit dissociation and protein unfolding.

## SUMMARY

The guanidine hydrochloride-induced subunit dissociation and unfolding of thermostable alanine racemase from *Bacillus stearothermophilus* have been studied by circular dichroism, fluorescence and absorption spectroscopies, and gel filtration. The overall process was found to be reversible: more than 75% of the original activity was recovered upon reduction of the denaturant concentration. In the range of 0.6-1.5 M guanidine hydrochloride, the dimeric enzyme was dissociated into a monomeric form, which was catalytically inactive. The monomeric enzyme appeared to bind the cofactor pyridoxal phosphate by a non-covalent linkage, although the native dimeric enzyme binds the cofactor through an aldimine Schiff base linkage. The monomer was mostly unfolded with a transition occurring in the range of 1.8 to 2.2 M guanidine hydrochloride.

## CHAPTER 2

### Limited Proteolysis of Alanine Racemase of *Bacillus stearothermophilus*

The primary structures of alanine racemases of *B. stearothermophilus* (12), *B. subtilis* (10), and two isozymes (DadB and Alr) of *Salmonella typhimurium* (8, 9) show considerable homology of about 33% to each other (12). Galakatos and Walsh (16) reported that both DadB and Alr ( $M_r$ 39,000 each) are cleaved at homologous positions with  $\alpha$ -chymotrypsin and subtilisin to produce non-overlapping peptides with  $M_r$ 28,000 and 11,000. They briefly mentioned that the *B. stearothermophilus* enzyme is similarly cleavable with subtilisin into two fragments (16). However, they had no comments about the activity of the thermostable alanine racemase after it was nicked. The author found that the nicked form of thermostable alanine racemase is still active.

## MATERIALS AND METHODS

### *Limited proteolysis of alanine racemase*

The enzyme (a final concentration, 4.1 mg/ml) was digested with subtilisin (0.1 mg/ml) at 37°C in 50 mM Tris-HCl buffer (pH 8.0) containing 0.01% 2-mercaptoethanol. Digestion was stopped by addition of phenylmethylsulfonyl fluoride (2 mM). The digested enzyme (10 $\mu$ g) was subjected to SDS-PAGE and stained for protein with Coomassie Brilliant Blue



R-250. Amount of stained protein in the gel was measured with a Shimadzu CS-900 dual wavelength TLC scanner.

### *Enzyme and protein assays*

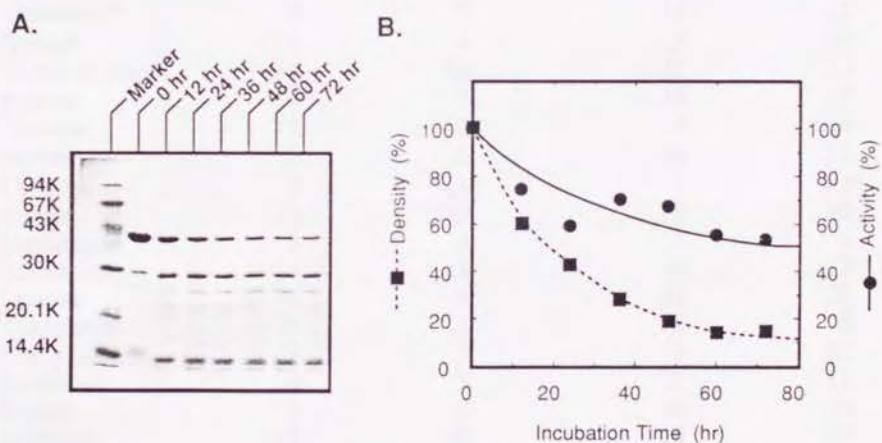
The enzyme activity was determined in D to L direction (Chapter 1). The protein concentration was determined by the same method of Bradford (21).

## RESULTS AND DISCUSSION

To characterize the nicked enzyme of *B. stearrowthermophilus*, we purified the enzyme from the *E. coli* recombinant cells as described previously (11,13), and digested it with subtilisin. Polyacrylamide gel electrophoresis in the presence of sodium lauryl sulfate (SDS-PAGE) showed that the enzyme composed of two identical subunits with  $M_r$ 43,000 was cleaved into two major fragments with  $M_r$ 14,000 (fragment F-1) and 29,000 (F-2) after 12 hr (Fig. 6A). Further incubation gave a new fragment of  $M_r$ 13,000 (F-1') with concomitant disappearance of F-1 (Fig 6A). We presumed that the primary cleavage occurred near  $^{264}\text{Tyr}$  based on the sequence homology to the two *S. typhimurium* enzymes (12), whose cleavage sites were identified (16). We isolated F-1 and F-2 fragments after digestion for 12 hr, by reversed phase high performance liquid chromatography (HPLC). The amino acid compositions of F-1 and F-2 were determined, and the result suggests that the enzyme is first cleaved by proteolysis around  $^{264}\text{Tyr}$  to F-1 and F-2 as expected (Table I).

The limited proteolysis was monitored at intervals by densitometric determination of the stained protein band in polyacrylamide gel (Fig. 6B). Approximately 85% of the native protein disappeared after incubation for 72 hr, but about 50% of the original activity remained. This suggests that about 15% of the native enzyme was unchanged, and the activity corresponding to 35% of the original activity was probably derived from the peptide fragments produced. The nicked forms of both *S. typhimurium* enzymes were only 3% as active as the corresponding native forms (16). Therefore, it is conceivable that the peptide fragments of the thermostable alanine racemase interact with each other more strongly to form a considerably active structure than that of the thermolabile enzyme, although no direct evidence is available yet.

We have constructed and expressed a mutant gene which tandemly encodes the two peptides corresponding to F-2 and F-1, and found that the gene products form a "fragmentary alanine racemase", which shows about 40% of the activity of the wild-type enzyme (Chapter3).



**Fig. 6.** Time course of limited proteolysis of alanine racemase. **A**, SDS-PAGE. **B**, Remaining activity of the digested enzyme. Density of the stained protein ( $M_r$ 43,000) in the gel were determined with a Shimadzu CS-900 dual wavelength TLC scanner.



**Table I.** Comparison of predicted and observed amino acid compositions of F-1 and F-2 fragments derived from alanine racemase after digestion with subtilisin.

Amino acid	Mol/mol of fragments			
	F-1		F-2	
	Predicted <sup>b</sup>	Observed <sup>c</sup>	Predicted <sup>b</sup>	Observed <sup>c</sup>
Aspartic acid	8	9	23	23
Threonine <sup>d</sup>	8	7	11	11
Serine <sup>d</sup>	4	4	12	10
Glutamic acid	12	12	22	24
Proline	7	7	19	17
Glycine	12	12	14	16
Alanine	7	6	30	32
Cysteine	3	nd <sup>e</sup>	1	nd <sup>e</sup>
Valine	11	10	18	18
Methionine	3	3	7	6
Isoleucine	14	13	10	12
Leucine	6	8	31	28
Tyrosine	4	3	10	8
Phenylalanine	3	3	13	12
Histidine	4	5	12	11
Lysine	3	3	10	10
Arginine	12	10	18	17

<sup>a</sup>The enzyme was digested in the same manner as described in Materials and Methods. The fragments were isolated by HPLC with Asahipak ODP-50 column after denaturation with guanidine hydrochloride.

<sup>b</sup>Calculated values on the assumption that the enzyme is cleaved to give F-1 (from <sup>265</sup>Gly to the CO<sub>2</sub>H-terminus) and F-2 (from <sup>1</sup>Met to <sup>264</sup>Tyr) fragments(12).

<sup>c</sup>Calculated values on the basis of fragment molecular weights: F-1, 14,000; F-2, 29,000.

<sup>d</sup>The values were obtained by extrapolation to zero hydrolysis time.

<sup>e</sup>Not determined.

## SUMMARY

Limited proteolysis of thermostable alanine racemase of *Bacillus stearothermophilus* was performed with subtilisin. SDS-PAGE showed that the enzyme composed of two identical subunits with  $M_r$ 43,000 was cleaved into two major fragments with  $M_r$ 14,000 (fragment F-1) and 29,000 (F-2). The amino acid compositions of F-1 and F-2 were determined, and the result suggests that the enzyme is first cleaved by proteolysis around  $^{264}\text{Tyr}$ . The limited proteolysis was monitored at intervals by densitometric determination of the stained protein band in polyacrylamide gel. Approximately 85% of the native protein disappeared after incubation for 72 hr, but about 50% of the original activity remained. This suggests that about 15% of the native enzyme was unchanged, and the activity corresponding to 35% of the original activity was probably derived from the peptide fragments produced. Therefore, it is conceivable that the peptide fragments of the thermostable alanine racemase interact with each other more strongly to form a considerably active structure than that of the thermolabile enzyme.

## CHAPTER 3

### Construction and Expression of Active Fragmentary Alanine Racemase of *Bacillus stearothermophilus*

Galakatos and Walsh (16) reported that both DadB and Alr isozymes of alanine racemase of *Salmonella typhimurium* suffered from limited proteolysis at two homologous positions by trypsin, chymotrypsin, or subtilisin, generating two non-overlapping polypeptides with  $M_r$  28,000 and 11,000, and a tetrapeptide, which was regarded as a hinge region. The DadB enzyme proteolyzed with chymotrypsin behaved as an associated form of two fragments, exhibited a far-UV CD profile identical with that of the native enzyme, and possessed 3% of the original catalytic activity. They also showed that the controlled subtilisin digestion of the thermostable enzyme of *B. stearothermophilus* gave a fragmentation pattern similar to that derived from Alr and DadB enzymes on SDS-PAGE. Although the *Bacillus stearothermophilus* enzyme shows only 35 and 31% sequence homology with DadB and Alr enzymes, respectively, local sequences including the active-site and hinge regions are well conserved.

To examine whether the two polypeptide fragments generated by limited proteolysis correspond to structural domains, the author attempted to prepare the two polypeptides by means of genetic engineering. The author has constructed a mutant alanine racemase gene of *B. stearothermophilus*, which contains a new set of termination and initiation codons in the position of the



gene corresponding to the putative hinge region of DadB and Alr racemases. The gene was expressed as an active alanine racemase composed of two dissimilar polypeptides.

## MATERIALS AND METHODS

### *Materials*

*Escherichia coli* JM109 and HB101, a 7-DEAZA sequencing kit, a DNA ligation kit, and T4 polynucleotide kinase were purchased from Takara Shuzo, Kyoto, Japan. Deoxycytidine 5'-[<sup>32</sup>P]triphosphate (400 Ci/mmol) was obtained from Amersham; the molecular weight determination kits for SDS-PAGE and gel filtration were from Pharmacia and Oriental Yeast, Japan, respectively; and a ZETAPREP 250 QAE cartridge was from CUNO, U.S.A.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 381A by the phosphoramidite method, and purified by HPLC with an Ultron NC-18 reversed-phase column. The following 6 oligonucleotides were prepared for construction of plasmid pARSD1 (AS-1 and AS-2) and pARSD2 (ARSD-1 and ARSD-2), and for sequencing (ASEQ-1 and ASEQ-2).

### *Construction of plasmid pARSD1*

The plasmid pMDalr3 (13) was enzymatically digested to isolate the 1.4 kb *Eco*RI-*Hind*III fragment containing the *B. stearothermophilus* alanine racemase gene, which was then digested with *Fsp*I. The oligonucleotides, AS-2 and phosphorylated AS-1, were annealed and ligated with the two *Fsp*I-

AS-1: 5'-GCA GTC ATA CTA AGA GCT C-3'

AS-2: 5'-CTA GGA GCT CTT AGT ATG ACT GC-3'

ARSD-1: 5'-CGA ATT CAA GGC AGC GAA AGC TTA TGA CGG  
CTC AAA CGG AGG AGT GGA TTG GGA CGA TTC  
CGA T-3'

ARSD-2: 5'-CGG AAT CGT CCC AAT CCA CTC CTC CGT TTG  
AGC CGT CAT AAG CTT TCG CTG CCT TGA ATT  
CGA GCT-3'

ASEQ-1: 5'-TAC ACG TCA AAA AAC TG-3'

ASEQ-2: 5'-GAA AGT GCT GCA GGC GG-3'

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digested fragments. These two linker-ligated fragments (0.5 kb and 0.9 kb, Fig. 7A) were separated by agarose gel electrophoresis and phosphorylated. The phosphorylated fragments were ligated with the *Eco*RI-*Hind*III-cut pKK223-3, then introduced into *E. coli* HB101. The transformants were selected by colony hybridization with <sup>32</sup>P-labelled AS-1 and AS-2 and analyzed by restriction mapping with *Eco*RI, *Hind*III, and *Sac*I. The resultant plasmid pARSD1 was directly sequenced on the denatured plasmid template using a 7-DEAZA sequencing kit and synthesized 3'-proximal primers, ASEQ-1 and ASEQ-2.

### *Construction of plasmid pARSD2*

The pairwise-complementary oligonucleotides ARSD-1 and ARSD-2 were kept at 94 °C for 1 min, rapidly cooled to 55 °C, and after 30 min to

room temperature. The *SacI*-*HindIII* fragment (5.5 kb) and the *HindIII*-*PvuI* fragment (0.5 kb) excised from pARSD1 were ligated with the annealed oligonucleotides to produce the plasmid pARSD2 (Fig. 7B). The construction of the plasmid was confirmed by restriction mapping and DNA sequencing.

### *Construction of plasmids pARND and pARCD*

The plasmid pARND encoding only the *N*-terminal fragment upstream from the hinge region was constructed by digestion of pARSD2 with *HindIII* and self-ligation of the longer fragment (5.5 kb), and selected by restriction mapping with *HindIII*. Similarly, the plasmid pARCD encoding only the *C*-terminal fragment downstream from the hinge region was constructed by digestion of pARSD2 with *EcoRI* and self-ligation of the longer fragment (5.1 kb). The structure of each plasmid was confirmed by DNA sequencing.

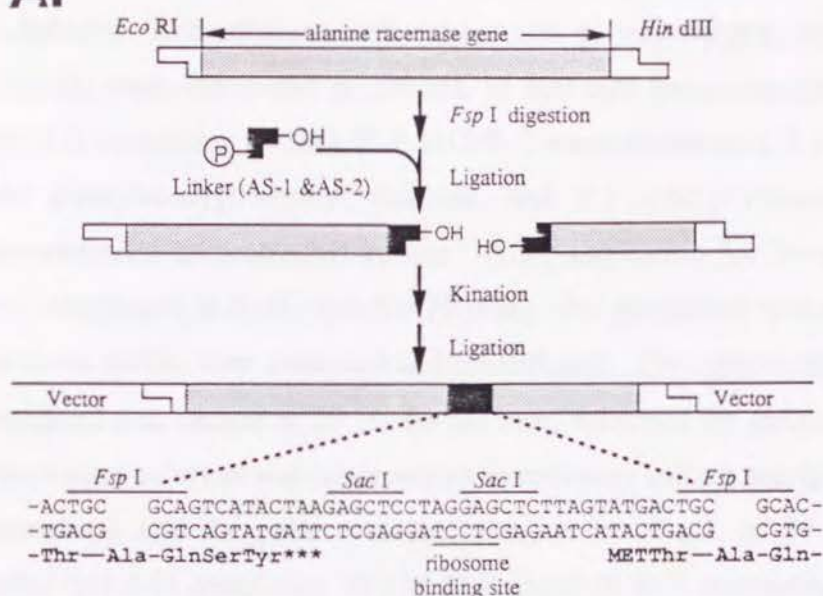
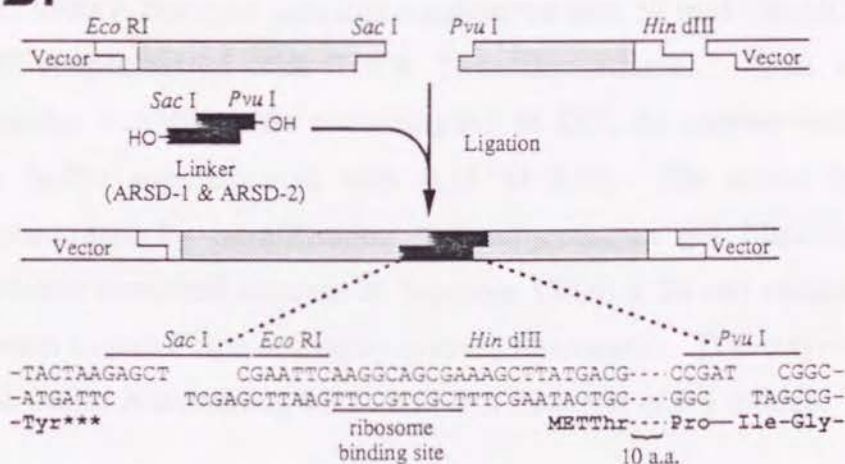
### *Cultivation of transformants*

All the four plasmids were introduced into *E. coli* JM109. Each transformant was cultured overnight in 5 mL of Luria-Bertani's broth supplemented with ampicillin (50 µg/ml). This culture was then inoculated into a fresh medium (500 mL). After incubation at 37 °C for 1.5 h, the expression of each enzyme was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested after further incubation for 8h.

### *Enzyme purification*

The cloned wild-type enzyme of *B. stearothermophilus* was purified as



**A.****B.**

**Fig. 7:** Construction scheme for plasmids pARSD1 (**A**) and pARSD2 (**B**). The closed area represents the inserted synthetic oligonucleotides; the length of DNA fragments shown is arbitrary.

described previously (11, 13). The fragmentary alanine racemase was purified as follows. Recombinant cells (about 100 g, wet weight) carrying plasmid pARSD2 were suspended in 200 mL of 100 mM potassium phosphate buffer (pH 7.2) containing 50 mM PLP, 0.02% 2-mercaptoethanol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone. After sonication for 20 min, the lysate was centrifuged at 8,000 rpm for 20 min. The precipitate was resuspended in the same buffer, then sonicated and centrifuged. The supernatant solution was combined and heated at 70 °C for 20 min, followed by centrifugation. The supernatant solution was subjected to ammonium sulfate precipitation (0-40% saturation), and the pellet was resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol (buffer A). After dialysis against buffer A, the sample solution was loaded on a ZETAPREP 250 QAE cartridge equilibrated with 50 mM Tris-HCl buffer (pH 8.0) supplemented with 0.01% 2-mercaptoethanol. After washing the cartridge with the buffer containing 0.1 M KCl, the enzyme was eluted with the buffer supplemented with 0.15 M KCl. The active fraction was concentrated by ultrafiltration and subjected to gel filtration with two tandemly connected columns of Superose 12 ( $\phi$ 1 x 30 cm) equipped on a Fast Protein Liquid Chromatography system (Pharmacia). The enzyme was eluted with buffer A containing 0.1 M KCl at a flow rate of 0.4 ml/min.

### *Enzyme and Protein Assays*

The enzyme activity was determined routinely in a reaction mixture containing D-alanine by monitoring the formation of NADH from NAD in the coupling system with L-alanine dehydrogenase (11). For assay of the racemase

activity from L-alanine, D-amino acid oxidase coupling assay (6) was employed. A unit of enzyme was defined as the amount that catalyzed the formation of 1  $\mu$ mol of a product per min. Protein concentration was determined with a BIO-RAD protein assay kit.

### *Spectrophotometric Measurements*

Absorption spectra were taken with a Shimadzu MPS-2000 recording spectrophotometer. CD measurements were performed with a Jasco J-600 recording spectropolarimeter at 25 °C with a 1-mm light path length cell under nitrogen atmosphere. Fluorescence measurements were carried out with a Hitachi MPF-4 spectrofluorophotometer.

## RESULTS

### *Construction and Expression of Fragmentary Alanine Racemase*

A previous limited proteolysis study on alanine racemases from Gram-positive and Gram-negative bacteria suggested that the enzyme subunit is composed of two domains (16). To examine whether the two polypeptides produced by the limited proteolysis correspond to the folding units of alanine racemase, the author has attempted to prepare a mutant gene which encodes two separate polypeptide fragments from the thermostable alanine racemase of *B. stearothermophilus* cleaved at the predicted hinge region (Pro258---Tyr268) (12). Hence, the author initially constructed plasmid pARSD1 containing a termination codon (TAA), a ribosome binding site (AGGAG),



and an initiation codon (ATG) at the *FspI* site occurring in the Thr269-Ala-Gln271 sequence close to the hinge region (Fig. 7A). However, the amount of the expressed product was very small, as judged from the enzyme activity in the cell extract (Table II) and the stained protein pattern in SDS-PAGE of the cell lysate. The author interpreted that this was probably due to insufficient translation by the presence of a palindromic structure and a short ribosome binding region in the synthetic oligonucleotide sequence newly inserted. Therefore, the author then redesigned a half of the inserted sequence in pARSD1 and replaced the ribosome binding sequence (AGGAG) by the one (AAGGCAGCGA) originally existing at the 5'-upstream region of the alanine racemase gene of *B. stearothermophilus* ((12); Fig. 7B). The recombinant cells carrying the plasmid pARSD2 thus constructed showed considerable alanine racemase activity (Table II). The mutant enzyme is designated hereafter as the fragmentary alanine racemase on the basis of its polypeptide composition as described below.

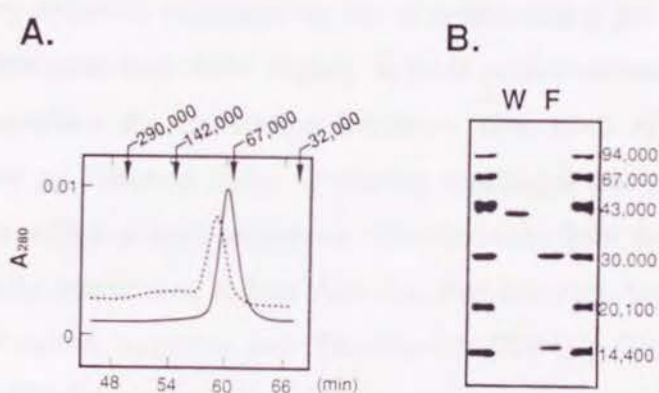
**Table II:** Alanine Racemase Activities in the Extracts of Recombinant cells.

recombinant cells	alanine racemase activity (units/mg)
<i>E. coli</i> JM109	0.07
<i>E. coli</i> JM109/pARSD1	1.72
<i>E. coli</i> JM109/pARSD2	16.9
<i>E. coli</i> JM109/pMDalr3 (Wild-type)	61.9

The author has also constructed two types of mutant plasmids (pARND and pARCD) encoding only either one of the *N*- and *C*-terminal fragments separated at the hinge region. However, the recombinant cells carrying either pARND or pARCD, which were grown under the same conditions with those carrying pARSD2, produced no proteins corresponding to the expected molecular weights (31,000 and 14,000, respectively) when examined by Western blotting of SDS-PAGE of their cell lysates using the antibody raised against the wild-type alanine racemase (data not shown), while the products from pARSD2 did react with the antibody (see below). The failure of the expression in separate host cells might be due to a rapid degradation of the separate fragment by cytoplasmic proteases after the translation, if any. It is also suggested that the two polypeptide fragments can fold into an active structure resistant to intracellular proteolytic digestion only when they are co-translated.

### *Purification and Structural Characterization of Fragmentary Alanine Racemase*

The author purified the mutant alanine racemase produced by *E. coli* JM109/pARSD2 in a large scale; from about 100 g of the wet cells, about 80 mg of the purified protein was obtained. The purified enzyme showed a symmetrical elution profile upon gel filtration under non-denaturing conditions, and the molecular size of the protein at the peak position was estimated to be about 84,000, which was slightly larger than that (80,000) of the wild-type enzyme (Fig. 8A). However, in the SDS-PAGE analysis, the mutant enzyme migrated as two dissimilar protein bands with approximate molecular sizes of 30,000 and 15,000 (Fig. 8B). The gel filtration in the



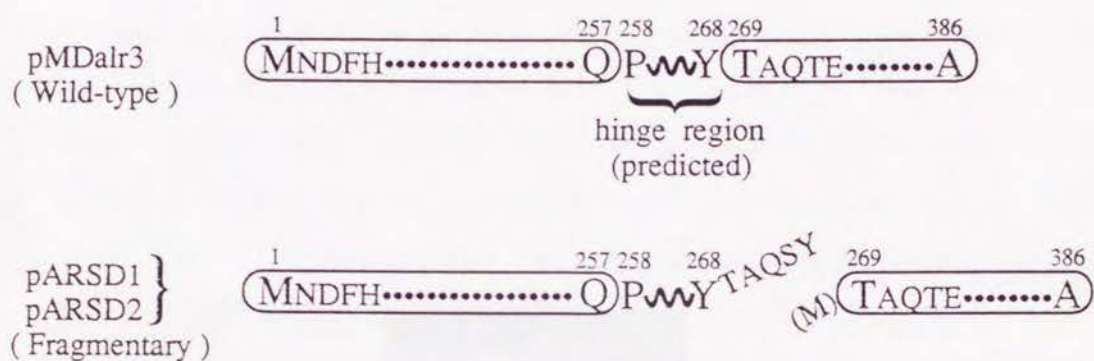
**Fig. 8:** A: Elution patterns of the wild-type (—) and fragmentary (---) alanine racemases in the non-denaturing gel filtration. Fifty micrograms of each protein were applied on two tandemly linked columns of Superose 12 and eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10  $\mu$ M PLP, 0.01% 2-mercaptoethanol, and 0.1 M KCl with a flow rate of 0.4 mL/min. The positions at which standard proteins were eluted are shown by arrows and their molecular weights. B: SDS-PAGE of the fragmentary (F) and wild-type (W) alanine racemases. Three micrograms of each protein were analyzed by SDS-PAGE with 13% acrylamide. Standard proteins are shown in both sides.



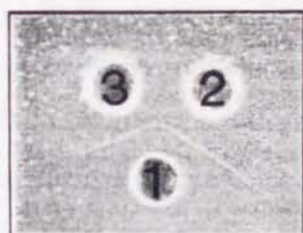
presence of 3 M guanidine hydrochloride also separated the mutant enzyme into two polypeptides (data not shown). These results show that the mutant enzyme has an  $\alpha_2\beta_2$ -type subunit structure, in contrast with the wild-type: alanine racemase of *B. stearothermophilus*, which is composed of two identical subunits (homodimer; (11)) with a molecular weight of about 43,000 ((12); Fig. 8B). Thus, the mutant enzyme was expressed in the recombinant cells as the author expected from the plasmid construction, and the fragmentary enzyme could be isolated as an associated form of the two polypeptide fragments. The notable difference between the molecular sizes of the wild-type and fragmentary enzymes estimated by the non-denaturing gel filtration suggests that these enzymes may differ slightly in their overall molecular shapes.

To confirm the translation initiation site, each of the polypeptides separated by gel filtration under denaturing conditions was subjected to Edman degradation with a protein sequencer. Ten residues from the *N*-terminus were unequivocally determined as Met-Asn-Asp-Phe-His-Arg-Asp-Thr-Trp-Ala- for the 30,000-dalton fragment and Thr-Ala-Gln-Thr-Glu-Glu-Trp-Ile-Gly-Thr- for the 15,000-dalton fragment. These sequences corresponded with the *N*-terminal sequence of the wild-type enzyme (12) and an internal sequence starting at position 269, respectively (Fig. 9). The translation initiator Met introduced in the 15,000-dalton fragment was removed in *E. coli*.

The fragmentary alanine racemase was indistinguishable from the wild-type enzyme upon Ouchterlony double-immunodiffusion analysis, forming a precipitation line with the antiserum against the wild-type enzyme, which fused completely with the line of the wild-type enzyme (Fig. 10). This indicates



**Fig. 9:** Schematic representation of the wild-type and fragmentary alanine racemases. Only several relevant amino acid residues in the terminal portions are shown.



**Fig. 10:** Ouchterlony double-immunodiffusion analysis of the fragmentary alanine racemase. Well **1**, the antiserum against the wild-type enzyme; well **2**, 4 mg of the wild-type enzyme; well **3**, 4 mg of the fragmentary enzyme.



that the antigenic structure on the molecular surface of the fragmentary enzyme is identical with that of the wild-type enzyme.

### *Catalytic Properties*

The specific activity of the purified fragmentary enzyme was about 600 units/mg protein under the standard assay conditions; the purified wild-type enzyme has a specific activity of about 1600 units/mg (11). To evaluate kinetically the decreased activity, the author performed steady-state kinetic analyses on the wild-type and fragmentary enzymes in both directions of the racemization reaction. In the D- to L-alanine direction, the  $K_m$  value for D-alanine was 1.8 mM, which was 2.2 times larger than that of the wild-type enzyme (0.82 mM) determined under the same assay conditions, and the  $V_{max}$  value was 930 units/mg, which was about a half of that of the wild-type enzyme (2000 units/mg). In the opposite direction (L- to D-alanine), the  $K_m$  values for L-alanine and the  $V_{max}$  values were as follows: the fragmentary enzyme, 4.5 mM and 2000 units/mg; the wild-type enzyme, 1.8 mM and 3800 units/mg. Thus, the fragmentary enzyme has lower activity and affinity for substrates than the wild-type enzyme. The catalytic efficiency of the fragmentary enzyme compared in terms of  $V_{max}/K_m$  values is about one fifth of that of the wild-type enzyme in both directions of the racemization reaction.

### *Thermostability*

The wild-type alanine racemase of *B. stearothermophilus* is a thermostable enzyme (11); it retains the full original activity on heating at 70 °C for 80 min. The fragmentary enzyme lost no activity by heating under the same conditions. However, when heated at 80 °C, the fragmentary enzyme

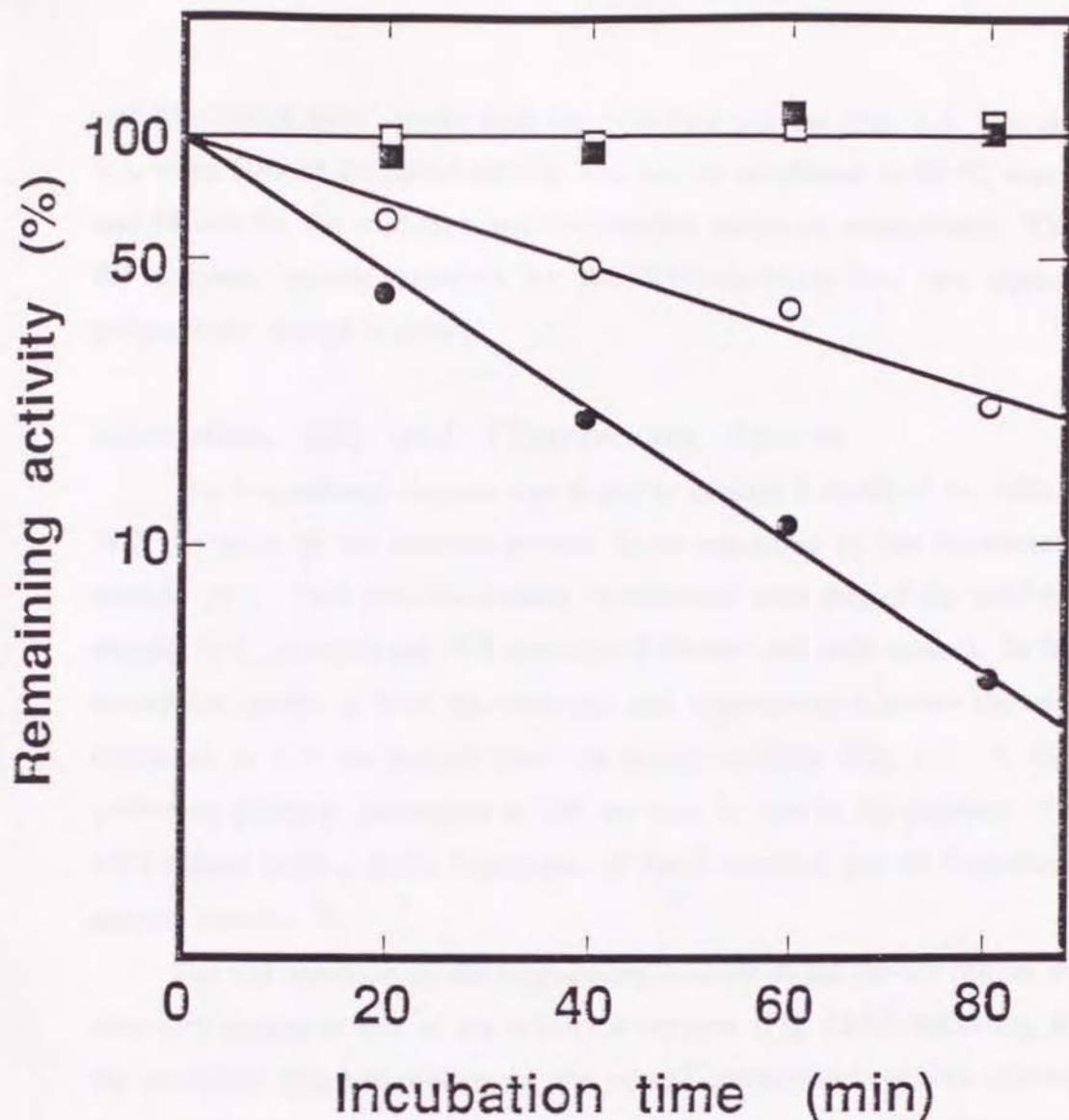


Fig. 11. Thermostability of the wild-type and fragmentary alanine racemases. The reaction mixture (1 mL) containing 5 mg of the wild-type or fragmentary enzyme, 100  $\mu$ mol of potassium phosphate buffer (pH 7.5), 10 nmol of PLP, and 0.1  $\mu$ g of 2-mercaptoethanol was incubated at 70 °C (squares) or 80 °C (circles). A 50- $\mu$ L aliquot was withdrawn at the indicated times, and the enzyme activity was measured with D-alanine as a substrate.  $\square$   $\circ$ , the wild-type enzyme,  $\blacksquare$   $\bullet$ , the fragmentary enzyme.

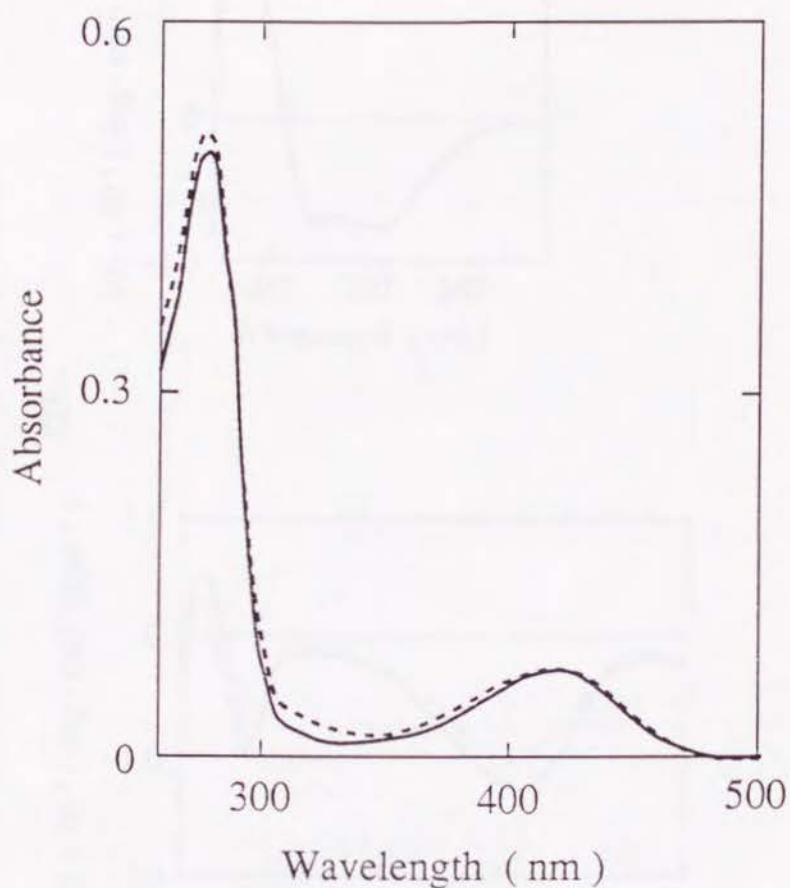
was inactivated more rapidly than the wild-type enzyme (Fig. 11). The time  $t_{1/2}$ , when 50% of the initial activity was lost by incubation at 80 °C, was 38 and 18 min for the wild-type and fragmentary enzymes, respectively. Thus, the enzyme became unstable by the fragmentation into two separate polypeptides, though slightly.

### *Absorption, CD, and Fluorescence Spectra*

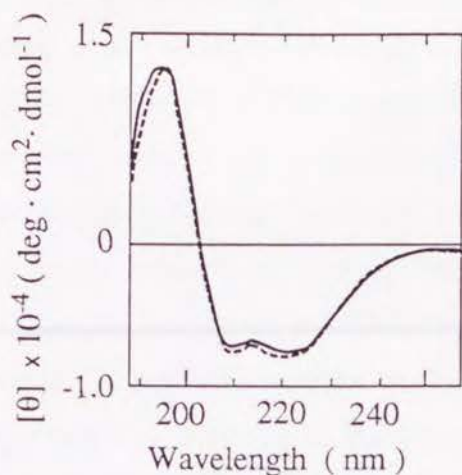
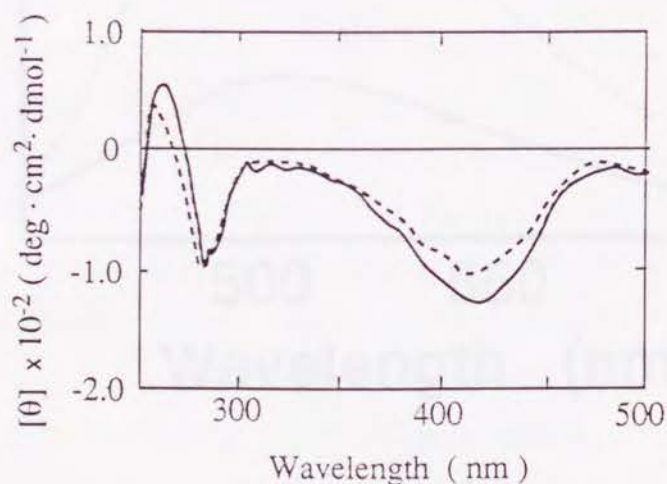
The fragmentary enzyme was found to contain 2 moles of the cofactor PLP per mole of the enzyme protein when measured by the fluorometric method (27). This cofactor content is identical with that of the wild-type enzyme (11), in which one PLP molecule is bound with each subunit. In fact, absorption spectra of both the wild-type and fragmentary enzymes showed a maximum at 420 nm derived from the bound cofactor (Fig. 12). A slight difference found in absorption at 280 nm may be due to the presence of an extra tyrosyl residue in the C-terminus of the N-terminal part of fragmentary enzyme (see Fig. 9).

The CD spectrum of the fragmentary enzyme in the far-UV region was also very similar to that of the wild-type enzyme (Fig. 13A), indicating that the secondary structure content and the overall conformation of both enzymes are virtually the same. Both enzymes exhibited a negative CD maximum at about 420 nm (Fig. 13B), corresponding to the absorption maximum due to the bound PLP (11). However, the molecular ellipticity at 420 nm of the fragmentary enzyme was apparently lower than that of the wild-type enzyme. This suggests that the conformation of the bound cofactor is slightly different between the two enzymes.





**Fig. 12.** Absorption spectra of the wild-type (—) and fragmentary (---) alanine racemases. Absorption spectra were taken in 10 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol at the enzyme concentration of 0.5 mg/mL.

**A****B**

**Fig. 13.** CD spectra of the wild-type (—) and fragmentary (---) alanine racemases. CD spectra were taken in 10 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol at the enzyme concentration of 0.1 mg/mL (**A**) and 3 mg/mL (**B**).

Relative fluorescence

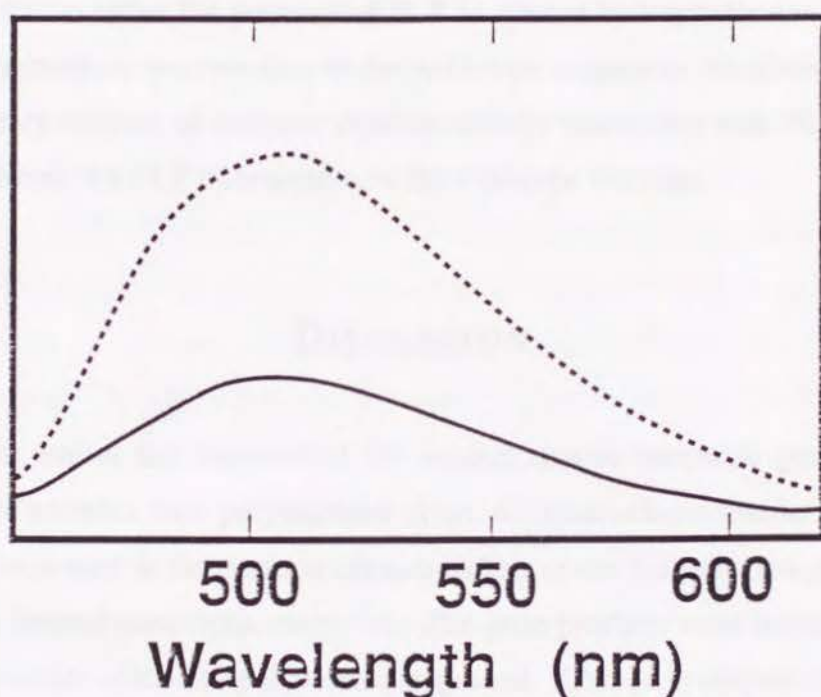


Fig. 14. Fluorescence emission spectra of the wild-type (—) and fragmentary (---) alanine racemases. Protein concentration was 0.1 mg/mL in 10 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol. Excitation at 420 nm. The emission due to free PLP in the buffer was subtracted.



The difference in the local environment around the bound cofactor was more evidently observed in fluorescence emission spectra of the bound PLP excited at 420 nm (Fig. 14). Interestingly enough, the cofactor bound with the wild-type enzyme is in a more quenched state than that with the fragmentary enzyme, emitting 2.4-fold less fluorescence at 510 nm. The higher fluorescence intensity of the bound cofactor in the fragmentary enzyme may be due to either the presence of PLP in a more hydrophobic environment in the fragmentary enzyme than in the wild-type enzyme or the absence in the fragmentary enzyme of aromatic residues closely interacting with PLP, which would quench the PLP fluorescence in the wild-type enzyme.

## DISCUSSION

The author has constructed the mutant alanine racemase gene which tandemly encodes two polypeptides from *B. stearothermophilus* enzyme subunit separated at the position corresponding to the hinge region predicted from the limited proteolysis study (16). The gene products were isolated as an active associate of the two polypeptide fragments. Change in enzyme activity is one of the most sensitive criteria to judge whether the overall conformation of enzyme proteins are affected deleteriously by mutation. Since the fragmentary enzyme was considerably active, its overall conformation is probably almost identical with that of the wild-type enzyme. In fact, the far-UV CD spectra of both enzymes were very similar. Conformational differences, if any, may be confined to a local region at or in the vicinity of the active site, judging from the slight difference in spectral properties of the

cofactor bound to the active site. It is suggested that the two polypeptide fragments correspond to structural folding units (domains) in the parental polypeptide chain of alanine racemase as defined by Levitt and Chothia (17).

Although the overall conformation of the fragmentary enzyme appeared to be identical with that of the wild-type enzyme, it showed only 40% of the activity of the native enzyme. The decreased activity of the fragmentary enzyme was accounted kinetically to a combined consequence of both the increase in the  $K_m$  values for substrates and the decrease in the  $V_{max}$  values. Galakatos & Walsh (28) showed by site directed mutagenesis studies that the hinge region in the DadB alanine racemase may play a role as a pivot for movement of the two domains during catalysis and may be located close to the active site, interacting with the bound PLP. Therefore, the decreased activity of the fragmentary enzyme relative to that of the native *B. stearothermophilus* enzyme could be due to either the presence of several extra residues in the hinge region or the discontinuity of the hinge sequence itself; both would constrain the possible functions of the flexible loop region. In accordance with this view, one of the DadB mutant enzymes, in which a fourth Gly was inserted into the intraloop tetrapeptide, showed a 5-fold drop in  $V_{max}/K_m$  (28) and the fragmentary *B. stearothermophilus* enzyme has a conformation of the bound cofactor slightly different from that in the wild-type enzyme.

The relative activity of the fragmentary enzyme to the wild-type enzyme was much higher than that of the clipped to the native DadB enzyme. This can be explained by an assumption that the thermostable enzyme has more extensive hydrophobic interdomain interactions than the DadB enzyme with less thermostability (12). The importance of hydrophobic interdomain



interactions for catalytic activities has been pointed out by studies on lactate dehydrogenase (29, 30).

The author could obtain neither the *N*-terminal nor *C*-terminal fragment alone from separate recombinant cells carrying pARND or pARCD. The failure in detection of the fragments expressed in separate host cells is probably ascribable to their proteolytic breakdown in the cells after the translation. The production of a similar *N*-terminal fragment from the DadB enzyme was also unsuccessful (28). These results suggest that the two polypeptide fragments can fold into an active structure only when they are co-translated. To examine whether the folding process of each fragment into the domain structure is cooperative each other during translation, establishment of the *in vitro* conditions for reversible resolution of the fragmentary enzyme into two polypeptides and their reconstitution into the active enzyme is certainly needed. Our future studies are addressed in this direction. The author are also trying to construct a series of mutant enzymes truncated in the hinge region to elucidate its catalytic function.

Although studies by limited proteolysis have provided a great deal of information on the domain structures of various proteins (e.g. Goldberg (31); Kirschner & Szadkowski (32); Edwards, et al. (33)), it is generally difficult to control the extent of limited proteolysis, and the generated polypeptide fragments are usually require purification in order for the folding process of individual fragments to be clarified. In this context, the fragmentation into domains by genetic engineering and their isolation as an active associate are very advantageous. The thermostable fragmentary alanine racemase is probably an interesting target to study the structure-function relationship of domains of proteins.



## SUMMARY

Limited proteolysis studies on alanine racemases suggested that the enzyme subunit is composed of two domains. We have constructed a mutant gene which tandemly encodes the two polypeptides of the *B. stearothermophilus* enzyme subunit cleaved at the position corresponding to the predicted hinge region. The mutant gene product purified was shown to be composed of two sets of the two polypeptide fragments, and was immunologically identical to the wild-type enzyme. The mutant enzyme, *i.e.* the fragmentary alanine racemase, was active in both directions of the racemization of alanine: the maximum velocity ( $V_{\max}$ ) was about half of that of the wild-type enzyme; the  $K_m$  value was about twice. Absorption and circular dichroism spectra of the fragmentary enzyme were similar to those of the wild-type enzyme. A single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli*, but no protein reactive with the antibody against the wild-type alanine racemase was produced. Therefore, it is suggested that the two polypeptide fragments can fold into an active structure only when they are co-translated and that they correspond to structural folding units in the parental polypeptide chain.

## CHAPTER 4

### Subunit Dissociation and Unfolding of Fragmentary Alanine Racemase of *Bacillus stearothermophilus*

The author studied dissociation and unfolding of the fragmentary alanine racemase of *Bacillus stearothermophilus* with guanidine hydrochloride, and characterized intermediates between the native and the denatured forms by circular dichroism and fluorescence spectroscopies, and by gel filtration chromatography.

The fragmentary enzyme also dissociates into two sets of a bimolecular complex of the two fragments (fragmentary subunits), and this occurs at lower concentrations of GdnHCl as compared with the wild-type enzyme. Dissociation and unfolding of the complex of the two fragments probably occur simultaneously. Since GdnHCl concentrations causing a series of these changes were similar to those of the wild-type enzyme, the secondary structure of the fragmentary enzyme probably is as stable as that of the wild-type enzyme.

## MATERIALS AND METHODS

All of the Materials and Methods are the same as that described in Chapter 1.

## RESULTS

### *Reversible Denaturation of Fragmentary Alanine Racemase*

When the fragmentary enzyme was incubated with L-alanine in 6 M GdnHCl at 37 °C for 30 min, D-alanine was not produced: the enzyme is inactive in 6 M GdnHCl. The inactivated enzyme was reactivated by dilution with 100 volumes of buffers (pH 8.0 - 10.5) to various extents according to pH of the buffers used (Fig. 15). The highest recovery was achieved around pH 10.0 and about 50% of the original activity was recovered, but effect of pH on the recovery was not so crucial as observed for the wild-type enzyme. When the denatured enzyme was dialyzed against 100 volumes of 50 mM Tris-HCl (pH 10.0) containing 10  $\mu$ M PLP and 0.01% 2-mercaptoethanol, about 50% of the original activity was recovered. Thus, two denatured fragments of the fragmentary enzyme, large and small fragments, are renatured either by dialysis or dilution to reduce the GdnHCl concentration.

### *Spectrophotometric Analysis of the Denaturation Process*

The absorption spectrum of the native fragmentary alanine racemase exhibits an absorption maximum at 420 nm in the visible region due to the aldimine linkage formed between PLP and the  $\epsilon$ -amino group of Lys39 in the large fragment (Chapter 3). Figure 16 shows that the peak at 420 nm was shifted to 390 nm with increase in GdnHCl concentrations (0 to 1.0 M), at lower concentration as compared with the wild-type enzyme (Chapter 1). This indicates that the aldimine bond is hydrolyzed to produce free PLP by addition of GdnHCl more easily than the wild-type enzyme. No further change in the



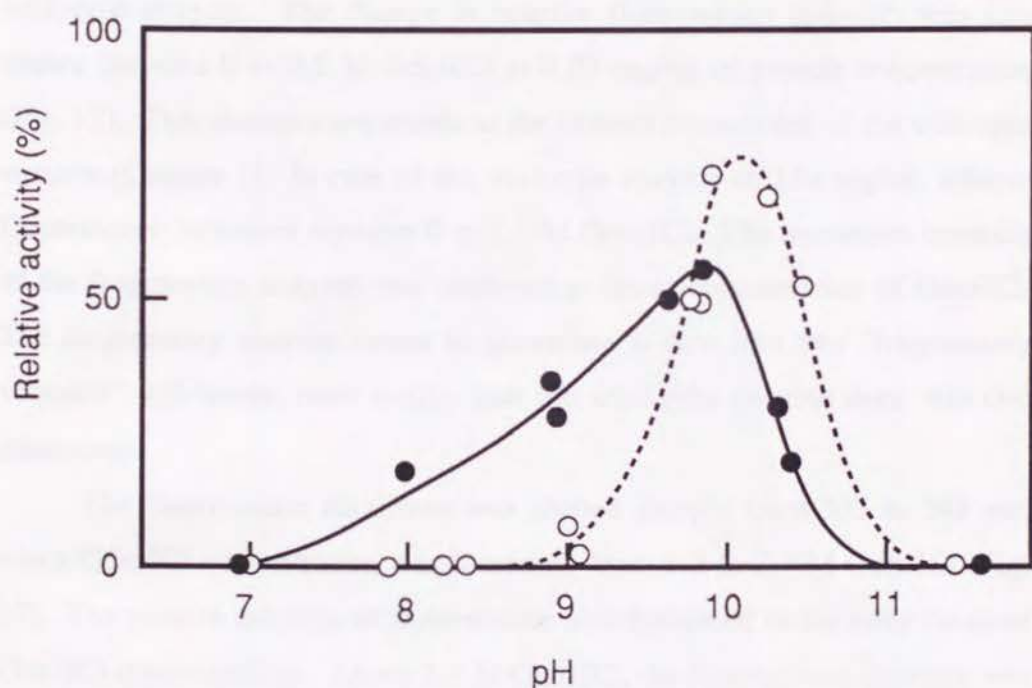


Fig. 15. Effect of pH on the renaturation of the fragmentary alanine racemase. The enzyme denatured with 6.0 M GdnHCl was renatured by 100-fold dilution with various kinds of buffers as described in Materials and Methods in Chapter 1. ○ wild-type enzyme, ● fragmentary enzyme.

spectrum was observed with GdnHCl of 1.6M or more: PLP occurs in an aldehyde form under the conditions (Fig. 16).

The author examined the fluorescence spectrum of the fragmentary enzyme derived from tryptophan residues. The fluorescence maximum moved gradually from 336 to 333 nm as GdnHCl concentration was increased (Fig. 17A). A transition midpoint was found around 0.5 M, lower than that of the wild-type enzyme. The change in relative fluorescence intensity was also shown between 0 to 0.5 M GdnHCl at 0.03 mg/ml of protein concentration (Fig. 17). This change corresponds to the subunit dissociation of the wild-type enzyme (Chapter 1). In case of the wild-type enzyme at 0.04 mg/ml, relative fluorescence increased between 0 to 1.3 M GdnHCl. The maximum intensity of the fragmentary enzyme was observed at lower concentration of GdnHCl. The fragmentary enzyme seems to dissociate at first into two "fragmentary subunits",  $\alpha\beta$ -forms, more easily than the wild-type enzyme does into two monomers.

The fluorescence maximum was shifted sharply from 333 to 348 nm, when GdnHCl concentration was increased from 1.2 to 2.4 M GdnHCl (Fig. 17). The relative intensity of fluorescence also decreased in the same range of GdnHCl concentrations. Above 2.4 M GdnHCl, the fluorescence intensity was not changed, as well as that of the wild-type enzyme. Physicochemical states of fully denatured wild-type and fragmentary enzymes are probably similar to each other. This is most probably the reason why the absolute fluorescence intensities of both enzymes in the presence of 6 M GdnHCl are similar. When we take the fluorescence intensity of the enzyme in the presence of 4 M GdnHCl as 100%, relative intensity of the maximum of both enzymes is

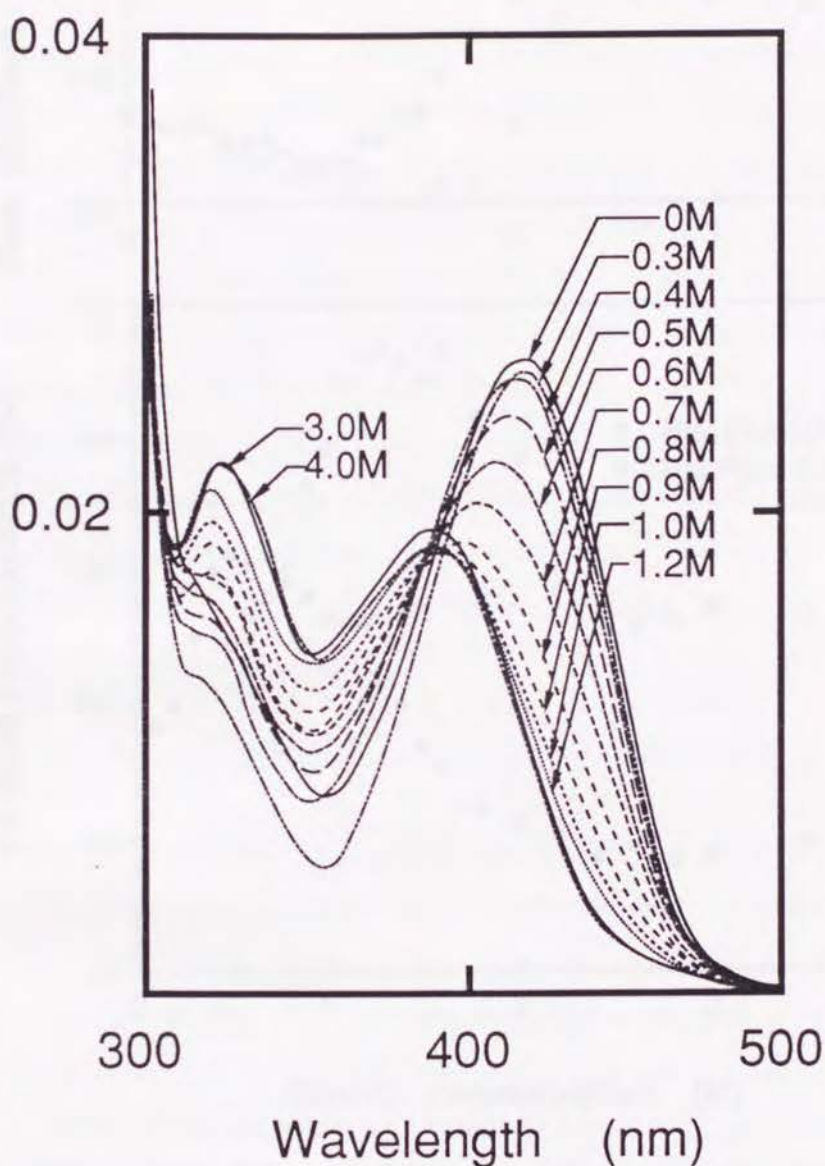


Fig. 16. Effect of GdnHCl concentrations on absorption spectra of the enzyme-bound PLP. The enzyme (final concentration, 0.18 mg/ml) was incubated with indicated concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then the absorption spectrum was measured.



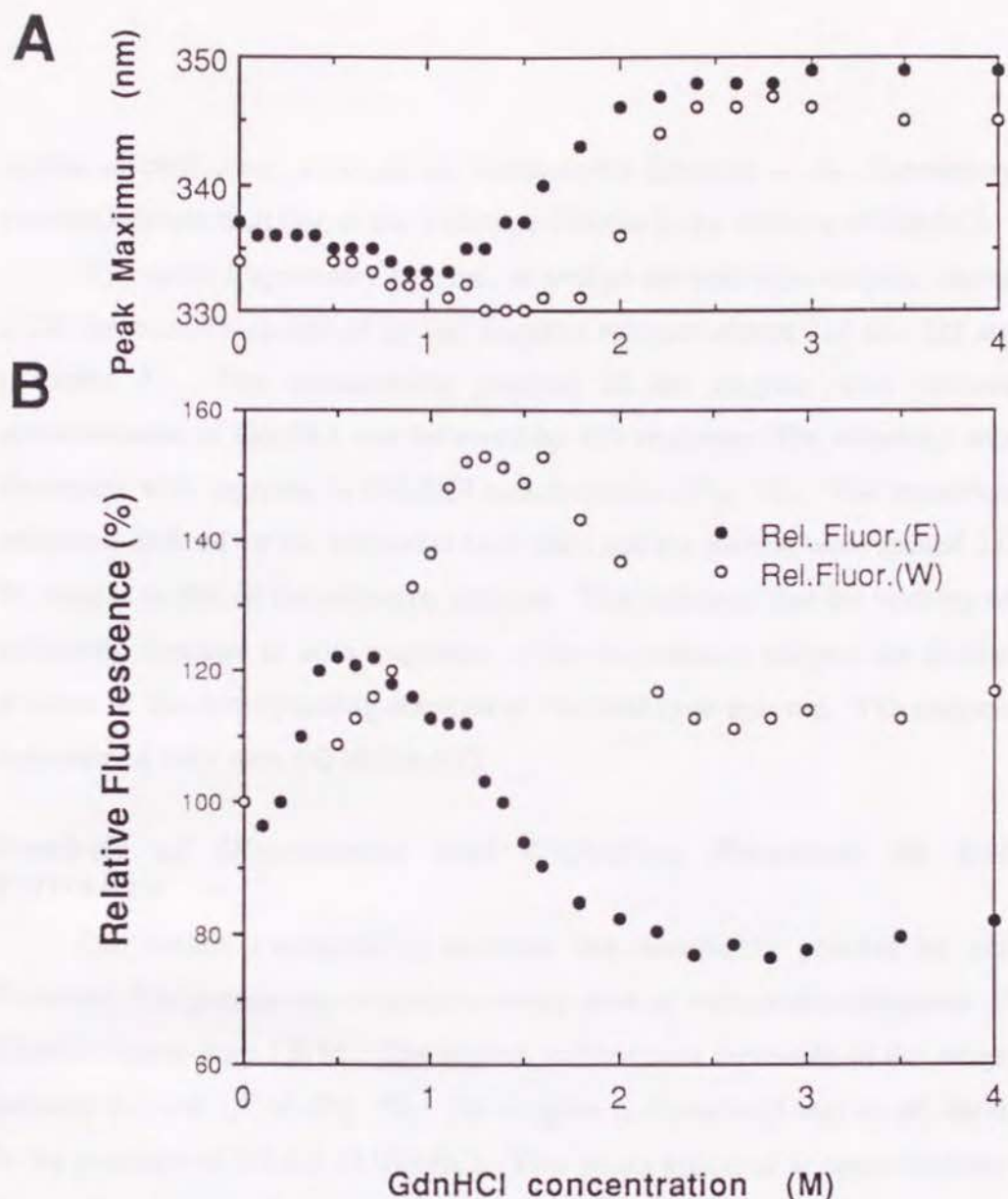


Fig. 17. Effect of GdnHCl concentrations on fluorescence spectra of the enzyme. The enzyme (final concentration, 0.1 mg/ml) was incubated with various concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then the fluorescence spectrum by excitation at 280 nm was measured. **A**, wavelength of the fluorescing maximum was plotted against GdnHCl concentrations. **B**, relative fluorescence intensity at 331 nm was plotted against GdnHCl concentrations. ○ wild-type enzyme, ● fragmentary enzyme.

similar to each other, although the fluorescence intensity of the fragmentary enzyme is lower than that of the wild-type enzyme in the absence of GdnHCl.

The native fragmentary enzyme, as well as the wild-type enzyme, shows a CD spectrum characterized by two negative minima around 210 and 222 nm (Chapter 3). The denaturation process of the enzyme with various concentrations of GdnHCl was followed by CD analyses. The ellipticity was decreased with increase in GdnHCl concentration (Fig. 18). The transition midpoints defined by the horizontal base lines and the plateau were around 2.0 M, similar to that of the wild-type enzyme. This indicates that the stability of secondary structure of both fragments of the fragmentary enzyme are similar to those of the corresponding domains of the wild-type enzyme. The enzyme is denatured fully with 6.0 M GdnHCl.

### *Analysis of Dissociation and Unfolding Processes by Gel Filtration*

The author attempted to examine the denaturing process by gel filtration. The protein was eluted as a single peak at various concentrations of GdnHCl lower than 1.8 M. The elution volume was increased in the range between 0.6 and 1.8 M (Fig. 19). The enzyme is dissociated into an  $\alpha\beta$ -form in the presence of 0.6-1.8 M GdnHCl. Two peaks appeared at concentrations higher than 2.4 M. The first peak corresponds to the large fragment, and the second one being the small fragment, as evidenced by SDS-PAGE (Chapter 5). Each elution volume was less than that expected from the mobility of marker proteins (Chapter 1). This indicates that two fragments were denatured under the conditions in contrast to the native enzyme, which was dissociated into native monomers under the same conditions. As the concentration was

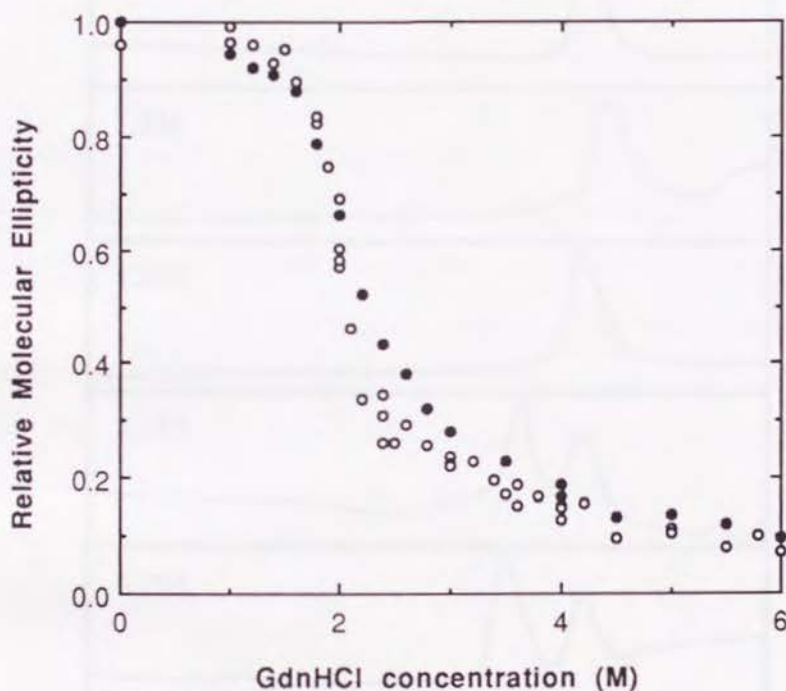


Fig. 18. Effect of GdnHCl concentrations on mean residue ellipticity at 222 nm of the fragmentary enzyme. The enzyme (final concentration, 0.1 mg/ml) was incubated with various concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 10 min, then CD was measured. The values indicated were relative when the value in the absence of GdnHCl was considered as 1.0. ○ wild-type enzyme, ● fragmentary enzyme.



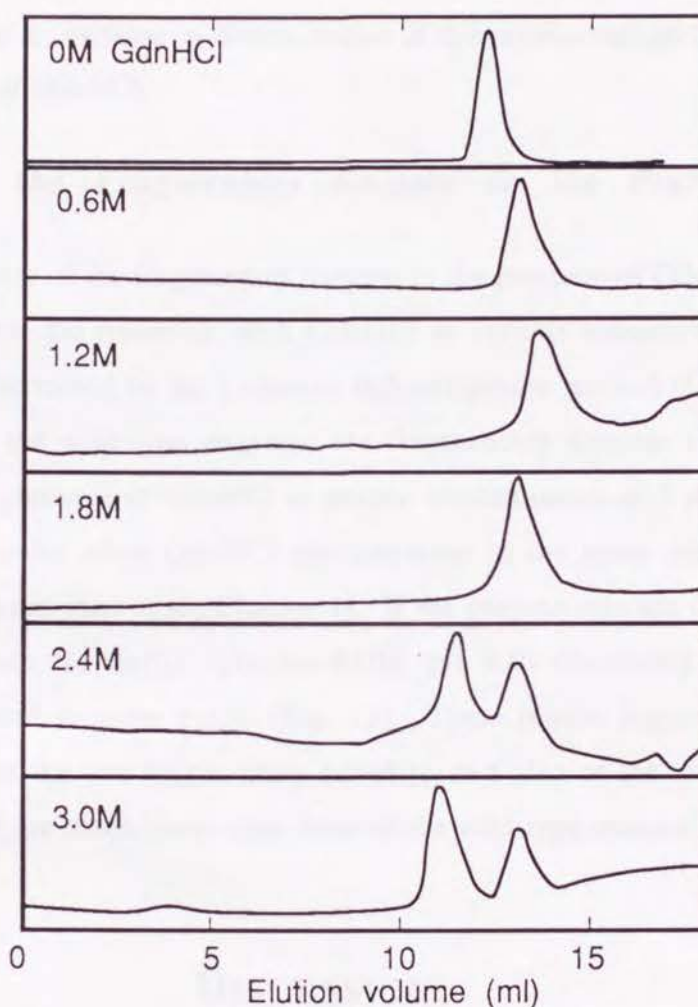


Fig. 19. Elution profiles of gel-filtration column chromatography of the fragmentary enzyme in the presence of various concentrations of GdnHCl. The enzyme (final concentration, 0.1 mg/ml) was incubated with indicated concentrations of GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20 °C for 30 min, then 50  $\mu$ l of the solution was applied to Superose 12 column as described in Materials and Methods in Chapter 1.

increased above 3.0 M, the elution volume was decreased further. This is probably due to an increase in Stokes radius of the protein unfolded at higher concentrations of GdnHCl.

### ***Activity of the Fragmentary Enzyme in the Presence of GdnHCl.***

The activity of the fragmentary enzyme in the presence of GdnHCl were measured. After the treatment with GdnHCl at various concentrations, the activity was determined by the L-alanine dehydrogenase method (Chapter 1). In contrast to the wild-type enzyme, the fragmentary enzyme showed no activity in the presence of GdnHCl at protein concentration of 5  $\mu\text{g/ml}$ , and restored no activity when GdnHCl concentration in the assay mixture was decreased (data not shown, see Chapter 1). If the enzyme solution in GdnHCl was diluted with the buffer (glycine-KOH, pH 9.0) containing PLP, the activity recovered to some extent (Fig. 15). These results suggest that the affinity between the two fragmentary subunits, and also of the fragmentary enzyme to PLP, are much lower than those of the wild-type enzyme.

## **DISCUSSION**

The fragmentary alanine racemase of *B. stearothermophilus* is unfolded by treatment with GdnHCl through two detectable phases in the same manner as the wild-type enzyme: phase 1, dissociation into two  $\alpha\beta$ -forms; phase 2, dissociation into two fragments and unfolding of each fragment. The author detected no distinct intermediate processes between the subunit dissociation and

the unfolding of the large and the small fragments. This indicates that each fragment has the different structure from that of the native enzyme after isolation (see Chapter 5). The two fragments separated by gel filtration at above 2.4 M GdnHCl were eluted faster than expected from their molecular weights. This is probably the reason why either fragment expressed in *E. coli* was isolated without success as described in Chapter 3.

The activity of the fragmentary enzyme after treatment with GdnHCl was not recovered unless PLP was added to the assay mixture. If the fragmentary enzyme was diluted against the buffer containing PLP, the activity recovered to some extent. These results probably reflect the lower affinity of the fragmentary enzyme to PLP than that of the wild-type enzyme. Tryptophan synthase from *Salmonella typhimurium* contains PLP at the interface between the two domains near the center of each  $\beta$  subunit as evidenced by X-ray crystallography (34). If alanine racemase also binds the cofactor in the inter-domain interface, the affinity of the enzyme to the cofactor is probably low.



## SUMMARY

The author showed that the fragmentary enzyme was dissociated into fragmentary subunits and unfolded through two detectable processes; phase 1, dissociation into two  $\alpha\beta$ -forms (fragmentary subunits); phase 2, dissociation into two fragments and unfolding of each fragment. At phase 1, absorption spectrum, fluorescence spectrum excited at 280 nm, and the profile of gel filtration were changed, and these occurred at lower concentration of GdnHCl than those of the wild-type enzyme. At phase 2, fluorescence and CD spectra were altered, and two peaks appeared in the profiles of gel filtration. Relatively low stability of the fragmentary enzyme as compared with the wild-type enzyme was probably due to the lower binding affinity between the two fragmentary subunits, because stability of the secondary structure was similar to that of the wild-type enzyme as judged from the results of CD analysis. In contrast to the wild-type enzyme, the fragmentary enzyme restored no activity in the assay mixture. If the fragmentary enzyme was diluted against the same buffer (glycine-KOH, pH 9.0) containing PLP, the activity recovered to some extent. This would reflect the lower affinity of the fragmentary enzyme to PLP than that of the wild-type enzyme.

## CHAPTER 5

### Reconstitution of Fragmentary Alanine Racemase of *Bacillus stearothermophilus*

The author have constructed a mutant gene which tandemly encodes the two polypeptide fragments corresponding to the putative domains (Chapter 3). The gene of the mutant enzyme composed of two sets of two fragments was over-expressed in *Escherichia coli*, and it shows about 40% activity of the wild-type enzyme and similar thermostability. The author also showed that the mutant enzyme, fragmentary alanine racemase, re-activated after denaturation with 6M guanidine hydrochloride (Chapter 4), which indicates that the two fragments in the fragmentary enzyme are the folding units both *in vivo* and *in vitro*, and are the putative domains in the wild-type enzyme.

The author here describes about the reconstitution of the fragmentary enzyme. The author has isolated the two fragments from the fragmentary enzyme in sufficient amounts to study their structure and function.

### MATERIALS AND METHODS

#### *Materials.*

Superose 12 packed column ( $\phi 1 \times 30$  cm) was purchased from Pharmacia; TSK-GEL G3000SWXL column ( $\phi 7.8 \times 300$  mm) was from Tosoh Corporation, Tokyo, Japan; guanidine hydrochloride (GdnHCl) (specially

prepared reagent grade) was from Nacalai Tesque, Kyoto, Japan. The fragmentary alanine racemase was purified from *E. coli* clone cells carrying a plasmid (pARSD2) prepared as described in CHAPTER 3. Alanine dehydrogenase of *B. stearothermophilus* was purified from the *E. coli* clone cells as described previously (13). The other chemicals were of analytical grade and purchased from Nacalai Tesque, Kyoto, Japan.

### ***Enzyme and Protein Assays.***

The enzyme activity was determined by following an increase in absorbance at 340 nm of a reaction mixture (1 ml) containing 100 mM glycine/KOH buffer (pH 9.0), 2.5 mM NAD<sup>+</sup>, 10 units of L-alanine dehydrogenase (11), 10 mM D-alanine, and enzyme at 50°C. A unit of alanine racemase is defined as the amount of enzyme that catalyzes the formation of 1  $\mu$ mol of L-alanine per min. Protein concentrations were determined by the method of Bradford (21). The absorption at 595 nm was read with an assay mixture (1 ml) of 0.8 ml of the sample and 0.2 ml of the concentrated dye reagent (Bio-Rad). To determine the concentration of the isolated fragments, the author used the following formula (35):  $\epsilon_M = (5500 \times N_{Trp} + 1400 \times N_{Tyr}) \times 1.05$ , in which  $N_{Trp}$  and  $N_{Tyr}$  are the number of tryptophan and tyrosine residues in alanine racemase, respectively:  $\epsilon_M$  for the large and small fragments are 3.50 and 1.02 cm<sup>-1</sup>M<sup>-1</sup>, respectively.



### ***Denaturation and Renaturation.***

The fragmentary enzyme was denatured with 6 M GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol. For renaturation, the enzyme solution was diluted with or dialyzed against 100 volumes of 50 mM glycine buffer (pH 10.0) containing 10  $\mu$ M PLP and 0.01 % 2-mercaptoethanol, and kept at 25°C for more than 12 h.

### ***Gel Filtration.***

Renatured enzyme solution was concentrated with an Ultracent 10 ultrafiltration unit (Tosoh Corporation, Japan). Gel filtration chromatography was done with a TSK-GEL G3000SWXL column equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.01 % 2-mercaptoethanol and 0.1 M potassium sulfoxide. Protein elution was monitored by measurement of the absorbance at 280 nm. An MW-Marker kit (Oriental Yeast, Japan) was used to estimate the molecular weight.

### ***Isolation of Subunits.***

The fragmentary alanine racemase denatured with 6 M GdnHCl at 25°C for 30 min was applied to a Superose 12 column ( $\phi$ 1 x 30 cm) equilibrated with 50 mM Tris/HCl buffer (pH 8.0) containing 10  $\mu$ M PLP, 0.01% 2-mercaptoethanol, 0.1 M KCl, and 4 M GdnHCl. The chromatography was done with a Pharmacia Fast Protein Liquid Chromatography (FPLC) system equipped with a Superose 12 column. Protein elution was monitored by measurement of the absorbance at 280 nm.

### ***Polyacrylamide Gel Electrophoresis.***

Analytical polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 0.1% SDS according to Laemmli (36). The gels were stained for protein with Coomassie Brilliant Blue R-250 at 70°C for 4 min and destained in 10% acetic acid. Relative molecular weight of polypeptides was determined by comparing with the relative mobilities of standard proteins.

### ***Spectrophotometric Measurements.***

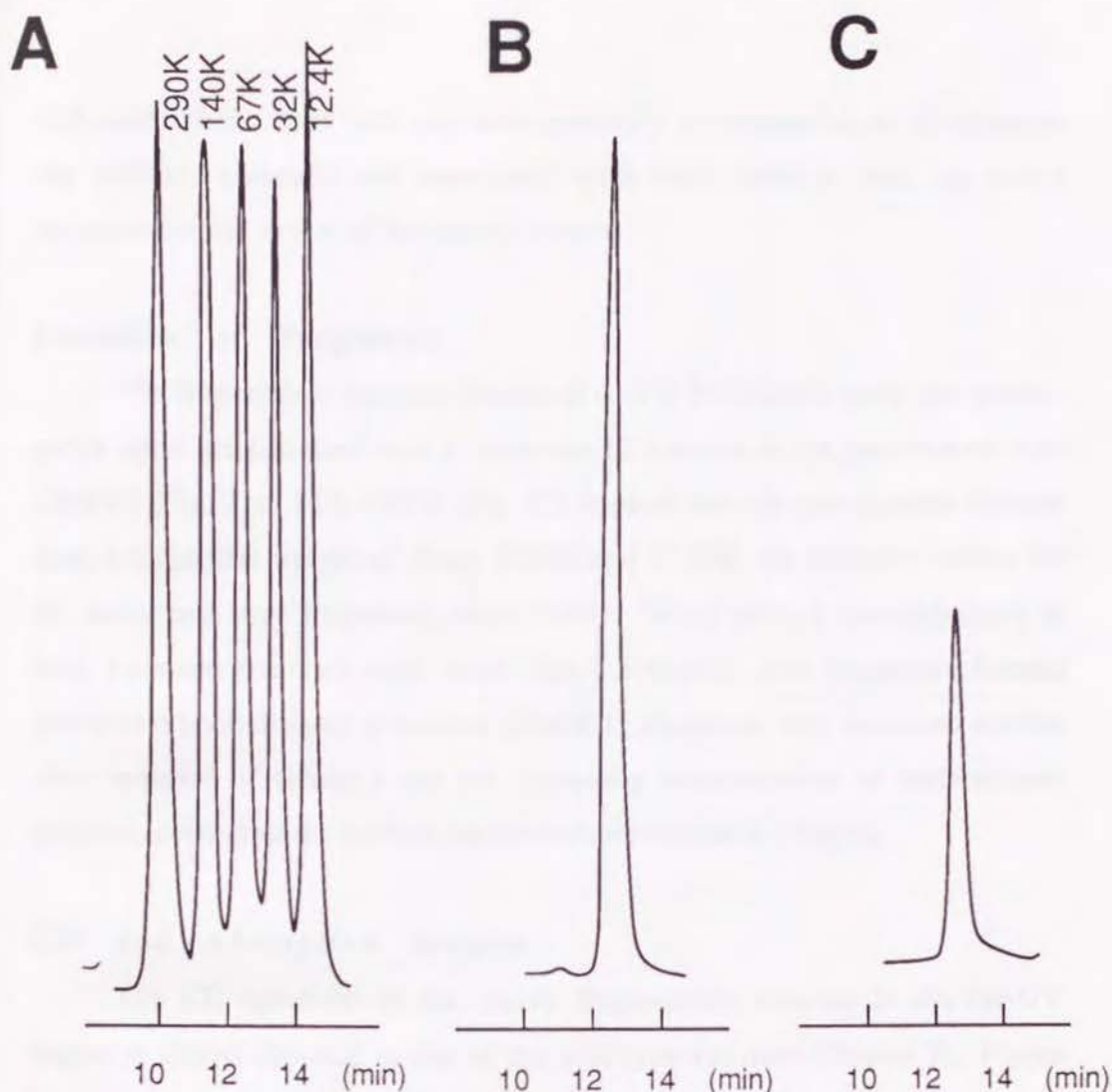
CD measurements were carried out with a JASCO J-600 recording spectropolarimeter at 25°C with a 1-mm light path length cell. The CD spectra were obtained at protein concentrations of 0.02-0.3 mg/ml in the far UV region (180 - 240nm) under nitrogen atmosphere. Absorption spectra were measured with a Beckman DU-50 spectrophotometer.

## **RESULTS**

### ***Reversible Denaturation of Fragmentary Alanine Racemase.***

The fragmentary enzyme was denatured by incubation with 6 M GdnHCl as shown by CD (Chapter 4). The denatured enzyme was reactivated by dilution or dialysis as shown in Chapter 4.

Gel filtration chromatogram of the renatured enzyme showed the same pattern as that of the native fragmentary enzyme (Fig 20). No peaks appeared at the position corresponding to the molecular weight of either small or large fragment. This shows that the two fragments correctly recognize and associate



**Fig. 20.** Gel filtration column chromatography of the renatured fragmentary alanine racemase. The denatured enzyme with 6 M GdnHCl was diluted by 80 fold with 50 mM Glycine-KOH buffer (pH 10.0) containing 0.01 % 2-mercaptoethanol. After renaturation, the protein solution was concentrated with an Ultracent 10 ultrafiltration unit (Tosoh Corporation, Japan) to the concentration of 1 mg/ml. An aliquot (50  $\mu$ l) was applied to a TSK-GEL G3000SWXL column. The low rate was 0.8 ml/min. **A**, molecular weight marker proteins; **B**, native fragmentary enzyme; **C**, renatured fragmentary enzyme.



with each other. Thus both fragments probably corresponding to the domains are refolded correctly and associated with each other to form an active structure similar to that of the native enzyme.

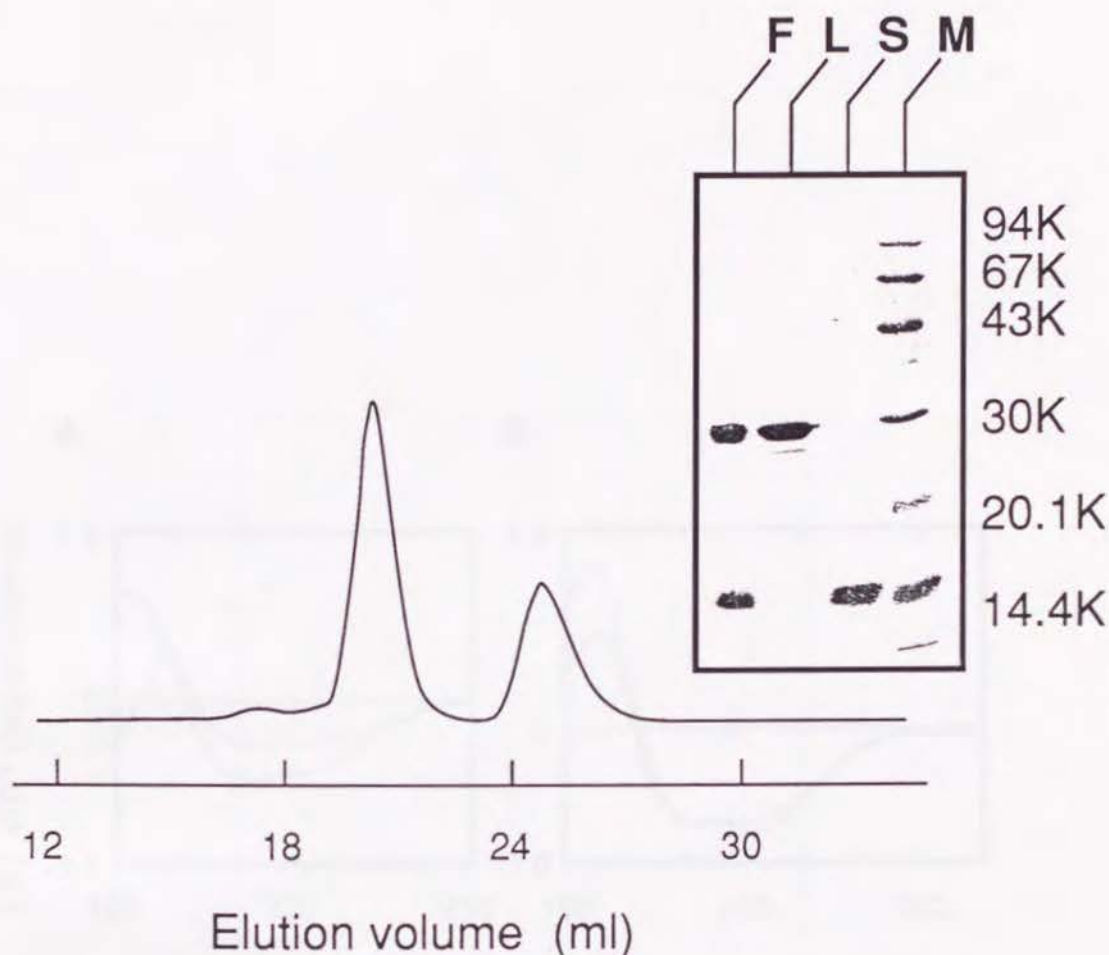
### ***Isolation of Fragments.***

The fragmentary enzyme denatured with 6 M GdnHCl gave two protein peaks upon gel filtration with a Superose 12 column in the presence of 4 M GdnHCl (Fig. 21). SDS-PAGE (Fig. 21) showed that the two peptides isolated have a molecular weight of about 15,000 and 31,000, the expected values for the small and large fragments, respectively. When protein concentrations in both fragment fractions were more than 0.1 mg/ml, both fragments formed precipitate upon dialysis to remove GdnHCl. However, they remained soluble after removal of GdnHCl and the following concentration of their diluted solution, even after the protein concentration reached 0.1 mg/ml.

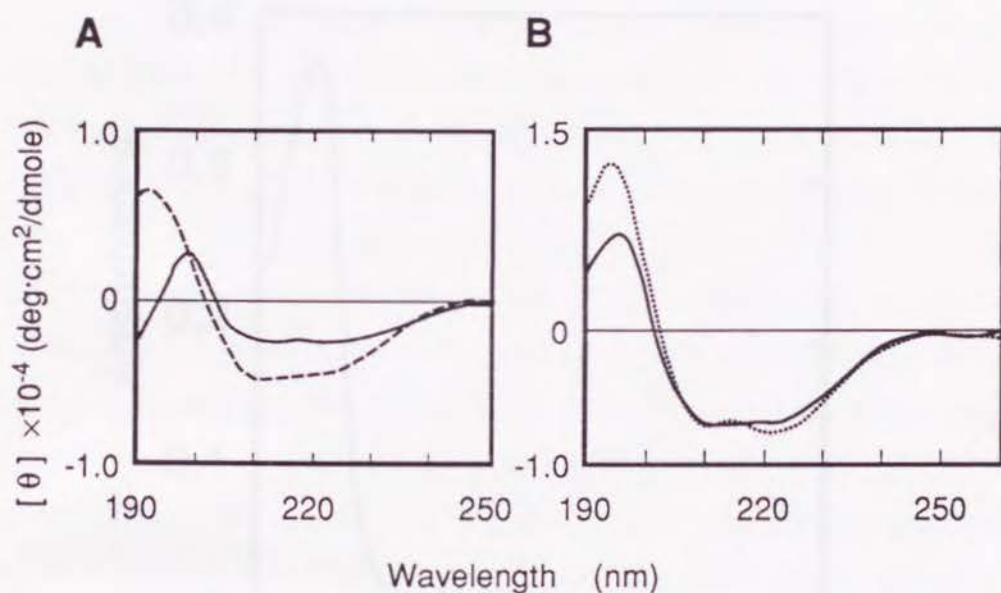
### ***CD and Absorption spectra.***

The CD spectrum of the native fragmentary enzyme in the far-UV region is almost identical to that of the wild-type enzyme (Chapter 3). Figure 22A shows that the CD spectra of the fragments isolated from the fragmentary enzyme are distinct from each other. However, the spectrum obtained by summation of the two spectra was close to that of the native fragmentary enzyme (Fig. 22B). These strongly suggest that each subunit is folded to give a secondary structure similar to that of the corresponding domain of the wild-type enzyme.

The PLP-bound lysine residue occurs in the large fragment of the fragmentary enzyme. The visible spectrum of the large fragment showed no

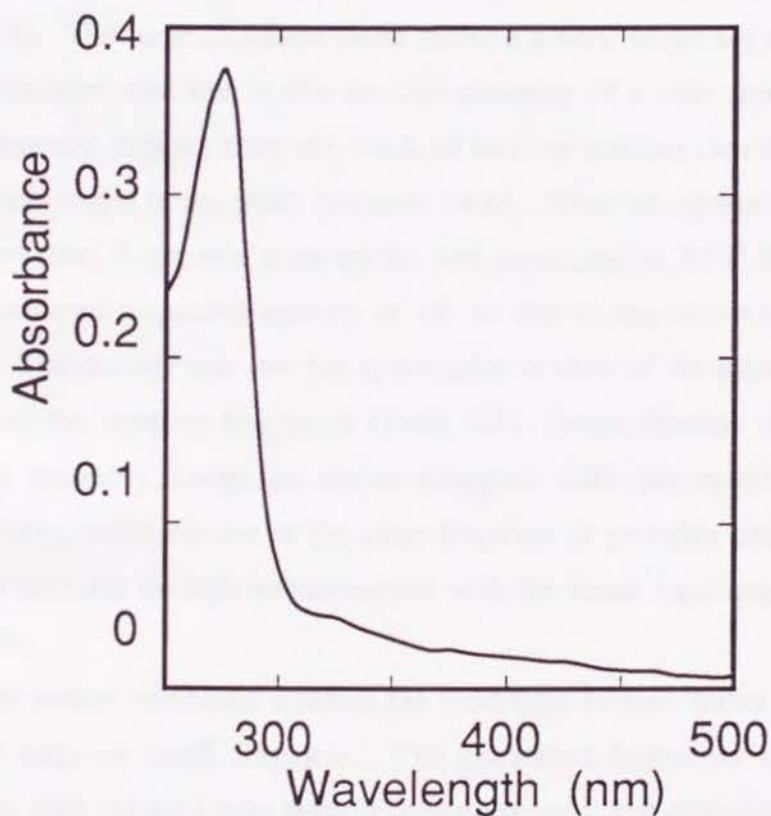


**Fig. 21.** Superose 12 gel filtration column chromatography of the fragmentary alanine racemase in the presence of 4 M GdnHCl. The enzyme (0.3 mg in 500  $\mu$ l) was incubated with 6 M GdnHCl in 10 mM potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol at 20°C for 30 min, then the enzyme was applied to two Superose 12 column ( $\phi$ 1 x 30 cm) tandemly connected. Protein elution was monitored at 280 nm. The flow rate was 0.05 ml/min. The insert shows SDS-PAGE of the two protein peaks: **L**, large fragment; **S**, small fragment. **F**, fragmentary enzyme **M**, molecular weight marker proteins.



**Fig. 22.** CD spectra of the isolated fragments (**A**). The fragments were dialyzed against 10 mM potassium phosphate buffer (pH 7.2) containing 10  $\mu$ M PLP and 0.01 % 2-mercaptoethanol prior to measurement. The ellipticity was expressed per decimole of protein. The protein concentrations were 0.18 mg/ml. -- large fragment, — small fragment. **B** shows the summation of the two spectra shown in **A**. — summation, ..... fragmentary enzyme.





**Fig. 23.** Absorption spectra of the isolated large fragment which was dialyzed against 10 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PLP and 0.01 % 2-mercaptoethanol. The protein concentration was 0.35 mg/ml.

characteristic absorption around 420 nm even in the presence of 10  $\mu$ M PLP (Fig. 23). This indicates that the large subunit can form a Schiff base with PLP only in the presence of the small fragment.

### *Activity of Fragments.*

Alanine racemase activity of the isolated fragments was determined (Table III). The large fragment alone showed a very slight activity, but the author considers that this is due to contamination of a trace amount of the small fragment, judging from the result of activity staining (see below). No activity was found in the small fragment alone. When an equimolar amounts of two isolated fragments were mixed and incubated at 25°C for 9 h, the mixture showed a specific activity of 1% of that of the native fragmentary enzyme. The activity was low but appreciable in view of the substantially no activity of the separate fragments (Table III). Some fraction of the large fragment probably forms an active complex with the small fragment. Alternatively, conformation of the large fragment is probably changed to be activated partially through an interaction with the small fragment during the incubation.

The author examined whether the wild-type subunit forms a complex with the large or small fragment. The denatured fragments were mixed separately with the wild-type subunit at various ratio, and dialysed to remove the denaturant by the method described in Materials and Methods. Then the proteins were separated by non-denaturing PAGE and stained by the enzyme activity. The author found that only the band corresponding to the dimeric wild-type enzyme showed activity (data not shown). The following possibilities were speculated; 1) binding affinity between the wild-type

**Table III** Alanine racemase activity of the isolated fragments.

	Alanine racemase activity	
	(Units/mg)	(%)
I. Fragmentary, Native	310	100
Renatured	150	48
II. Large fragment (31K)	0.1	0.0
Small fragment (15K)	0.0	0.0
III. 31K + 14K, only mixed	3.1	1.0

subunits is much higher than that between the wild-type subunit and the either fragment, and 2) the complex of the wild-type subunit and the either fragment has no activity.

## DISCUSSION

Both subunits constituting the fragmentary enzyme were readily isolated by gel filtration after denaturation with 6 M GdnHCl. They were spontaneously refolded into an active form upon removal of GdnHCl. Each subunit alone was also refolded to give a secondary structure probably similar to that in the wild-type enzyme. These suggest that both subunits correspond to independently folding units previously named as "globules" by Goldberg



(31). Högberg-Raibaud and Goldberg (37) isolated the two fragments of  $\beta$  chain of tryptophan synthase nicked by trypsin and demonstrated that the two fragments are considered as independently folding region corresponding to intermediates of the folding of the intact protein. The folding of the wild-type alanine racemase probably proceeds through the folding of these globules.

The visible spectrum of the large fragment isolated showed that it does not bind with PLP to form Schiff base even by addition of 10  $\mu$ M PLP. In fact, the large fragment alone showed no significant activity, although it was activated a little by incubation with the small fragment. Both fragments need to be folded together in order to form an active structure comparable with the native fragmentary enzyme. The fine protein conformation necessary for the catalytic activity is probably formed only through an interchain association of both fragments.

## SUMMARY

The two polypeptides (i.e. large and small fragments) were isolated from the fragmentary alanine racemase by Superose 12 gel filtration in the presence of 4 M guanidine hydrochloride. After removal of the denaturant, the two fragments were reconstituted and reactivated as judged from the results of gel-filtration analysis and the activity measurement. The spectra obtained by summation of the CD spectra of the isolated subunits was close to that of the native fragmentary enzyme. The lysine residue to which PLP is bound in the wild-type enzyme occurs in the large subunit of the fragmentary enzyme. The visible spectrum of the large subunit indicates that PLP is not bound to it. The large subunit alone showed no significant activity, but it was activated by incubation with the small subunit. These indicate that the two fragments are the units of the folding, and that both fragments need to be folded together in order to form an active structure.

## CONCLUSION

In this thesis, the author studied structure and function of thermostable alanine racemase from *Bacillus stearothermophilus*.

The guanidine hydrochloride (GdnHCl)-induced subunit dissociation and unfolding of thermostable alanine racemase from *B. stearothermophilus* were studied by circular dichroism (CD), fluorescence and absorption spectroscopies, and gel filtration. The overall process was found to be reversible: more than 75% of the original activity was recovered upon reduction of the denaturant concentration. In the range of 0.6-1.5 M GdnHCl, the dimeric enzyme was dissociated into a monomeric form, which was catalytically inactive. The monomeric enzyme appeared to bind the cofactor pyridoxal phosphate (PLP) by a non-covalent linkage, although the native dimeric enzyme binds the cofactor through an aldimine Schiff base linkage. The monomer was mostly unfolded with a transition occurring in the range of 1.8 to 2.2 M GdnHCl.

The author examined limited proteolysis of thermostable alanine racemase of *B. stearothermophilus* with subtilisin. SDS-PAGE showed that the enzyme composed of two identical subunits with  $M_r$ 43,000 was cleaved into two major fragments with  $M_r$ 14,000 (fragment F-1) and 29,000 (F-2). The amino acid compositions of F-1 and F-2 were determined, and the result suggests that the enzyme is first cleaved by proteolysis around <sup>264</sup>Tyr. The limited proteolysis was monitored at intervals by densitometric determination of the stained protein band in polyacrylamide gel. Approximately 85% of the native protein disappeared after incubation for 72 hr, but about 50% of the original activity remained. This suggests that about 15% of the native enzyme



was unchanged, and the activity corresponding to 35% of the original activity was probably derived from the peptide fragments produced. Therefore, it is conceivable that the peptide fragments of the thermostable alanine racemase interact with each other more strongly to form a considerably active structure than that of the thermolabile enzyme.

Limited proteolysis studies on alanine racemases suggested that the enzyme subunit is composed of two domains. The author constructed a mutant gene which tandemly encodes the two polypeptides of the *B. stearothermophilus* enzyme subunit cleaved at the position corresponding to the predicted hinge region in Chapter 3. The mutant gene product purified was shown to be composed of two sets of the two polypeptide fragments, and was immunologically identical to the wild-type enzyme. The mutant enzyme, *i.e.* the fragmentary alanine racemase, was active in both directions of the racemization of alanine: the maximum velocity ( $V_{max}$ ) was about half of that of the wild-type enzyme; the  $K_m$  value was about twice. Absorption and circular dichroism spectra of the fragmentary enzyme were similar to those of the wild-type enzyme. A single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli*, but no protein reactive with the antibody against the wild-type alanine racemase was produced. Therefore, it is suggested that the two polypeptide fragments can fold into an active structure only when they are co-translated and that they correspond to structural folding units in the parental polypeptide chain.

The author examined the dissociation and unfolding process of the fragmentary alanine racemase. The author showed two detectable processes; phase 1, dissociation into two  $\alpha\beta$ -forms (fragmentary subunits); phase 2, dissociation into two fragments and unfolding of each fragment. At phase 1,

absorption spectrum, fluorescence spectrum excited at 280 nm, and the profile of gel filtration changed, and these occurred at lower concentration of GdnHCl than those of the wild-type enzyme. At phase 2, fluorescence and CD spectra were altered, and two peaks appeared in the profiles of gel filtration. Relatively low stability of the fragmentary enzyme as compared with the wild-type enzyme was probably due to the lower binding affinity between the two fragmentary subunits, because stability of the secondary structure was similar to that of the wild-type enzyme as judged from the CD analysis. In contrast to the wild-type enzyme, the fragmentary enzyme restored no activity in the assay mixture. If the fragmentary enzyme was diluted against the same buffer (glycine-KOH, pH 9.0) containing PLP, the activity recovered to some extent. This probably reflects the lower affinity of the fragmentary enzyme to PLP than that of the wild-type enzyme.

The author isolated the large and the small fragments from the fragmentary alanine racemase after denaturation with 6 M GdnHCl. The two fragments reconstituted and reactivated after the concentration of the denaturant reduced as judged from the results of gel-filtration analysis and the activity measurement. The spectra obtained by summation of the CD spectra of the isolated subunits was close to that of the native fragmentary enzyme. The lysine residue to which PLP is bound in the wild-type enzyme occurs in the large subunit of the fragmentary enzyme. The visible spectrum of the large subunit indicates that PLP is not bound to it. The large subunit alone showed no significant activity, but it was activated by incubation with the small subunit. These indicate that the two fragments are the units of the folding, and that both fragments need to be folded together in order to form an active structure.



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